

Reprint Compendium

Bio-protocol Selections 2021

Plant Science

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Foreword

We are pleased to launch the second Bio-protocol series of reprint collections, comprising some of the most used protocols published in 2019 and 2020 in several research areas. This collection focuses on plant science.

Established in 2011 by a group of Stanford scientists, Bio-protocol's mission is to improve research reproducibility and usability through the publication of high quality step-by-step peer-reviewed life science protocols. Bio-protocol invites contributions from authors who have published methods in brief, as part of other research articles, and who might want to provide more detail to facilitate use by others. A survey carried out in 2018 showed that, of more than 2300 users who had followed a protocol published in Bio-protocol, 91% (2166 users) were able to successfully reproduce the method they tried.

In this reprint collection, we have selected 26 plant science research protocols published in Bio-protocol in 2019 and 2020 that we consider the most highly used. We base this classification on metrics such as the number of times a protocol was viewed or downloaded by unique users, and the number of citations it has received in other publications.

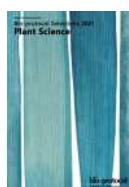
Hopefully, you will find this collection intriguing and visit www.bio-protocol.org to check out the entire archive of protocols. Please feel free to email us (eb@bio-protocol.org) with feedback, and please consider contributing a protocol to Bio-protocol in the future.

The Bio-protocol Editorial Team

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Image from the protocol "**Efficient Agrobacterium-mediated Transformation of the Elite–*Indica* Rice Variety Komboka**". *Bio-protocol* 10(17): e3739. DOI: 10.21769/BioProtoc.3739.

***In vitro* Protein-DNA Binding Assay (AlphaScreen® Technology)**

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[Abstract] Identification of specific DNA binding sites of transcription factors is important in understanding their functions. Recent techniques allow us to investigate genome-wide *in vivo* binding positions by chromatin immunoprecipitation combined with high-throughput sequencing. However, to further explore the binding motifs of transcription factors, in-depth biochemical analysis is required. Here, we describe an efficient protocol of protein-DNA interactions based on a combination of our *in vitro* transcription/translation system and AlphaScreen® technology. The *in vitro* transcription/translation system supports an efficient and quick way of protein synthesis by alleviating cumbersome cloning steps. In addition, AlphaScreen® system provides a highly sensitive, quick, and easy handling platform to investigate the protein-DNA interactions *in vitro*. Thus, our method largely contributes to comprehensive analysis of the biochemical properties of transcription factors.

Keywords: Transcription factor, *In vitro* transcription/translation, FLAG-tag, Protein-DNA interaction, AlphaScreen, DNA binding

[Background] Upon exposure to abiotic and biotic stresses, major transcriptional changes are induced in plants to help them adapt to these environmental stimuli. Transcriptional regulation is important not only for stress responses but also for plant development. In general, one transcription factor regulates an array of target genes by recognizing its corresponding cis-regulatory elements. Thus, to study the physiological role of transcription factors, identification of their specific binding sequences is a major challenge. Recently, chromatin immunoprecipitation combined with high-throughput sequencing (ChIP-seq) has enabled researchers to determine the genome-wide locations of a transcription factor-DNA interaction. However, the precise binding capability should be further confirmed by in-depth biochemical experiments.

For the past several decades, electrophoretic mobility shift assay (EMSA) and transient reporter assay using viable cells have been widely used to determine protein-DNA interactions. For EMSA, a protein-DNA complex is subjected to electrophoresis followed by the autoradiographic or immunodetection of the band shift. For transient assay, protoplasts or tissues are transformed with a plasmid that consists of a promoter sequence harboring candidate cis-regulatory elements fused with a reporter gene encoding such as luciferase or green fluorescent protein (Chalfie et al., 1994). Although these are powerful methods to investigate the direct binding site of a transcription factor and its transcriptional activity, they are time-consuming and thus cannot be applicable to a comprehensive analysis.

On the other hand, Amplified Luminescence Proximity Homogenous Assay (Alpha) technology is an emerging alternative method to effectively detect protein-DNA interaction (AlphaScreen®). AlphaScreen assay uses two types of small beads (250 nm in diameter), Acceptor and Donor beads, which are specifically designed to associate with either a target protein fused with a protein tag or a biotinylated cis-regulatory element. When the protein-DNA interaction occurs, illumination at 680 nm releases singlet oxygen from donor beads, which subsequently transfers energy to acceptor beads to generate light at 520-620 nm. Since the half-life of singlet oxygen is only 4 μ sec, the interaction is not detected without close proximity (see also web site: www.perkinelmer.com/alphascreen). Since AlphaScreen® shows high sensitivity and low background in a microplate format, it can adapt to a high-throughput screening platform.

Compared to EMSA and transient assay, AlphaScreen® is considerably easier to handle and quicker for the detection of protein-DNA interaction. Moreover, we have developed a highly efficient way to synthesize proteins *in vitro* (Nomoto and Tada, 2018a and 2018b). This method enables us to synthesize proteins that are difficult to obtain in cell-based production systems such as *E. coli*. Furthermore, this method is highly time efficient because the DNA template for *in vitro* transcription can be made by the second round of PCR using any type of cloning vectors. To take these advantages, we describe a protocol from protein synthesis to AlphaScreen® assay for detecting protein-DNA interactions. We confirm that our method contributes to the understanding of biological roles of transcription factors function in diverse signaling pathways.

Materials and Reagents

1. Aluminum foil
2. 1.5 ml microcentrifuge tubes
3. 0.2 ml 8 strip PCR tube and cap strips
4. 5'-biotinylated and non-biotinylated 50 base single-strand DNA and complementary unmodified 50 base single-strand DNA (Eurofins, Japan)
5. Plasmid containing cDNA region of the gene of interest
6. Gene-specific primers for 1st and 2nd PCR (see Table 1)
7. Ultrapure water (Milli-Q water)
8. KOD-Plus-Neo (including 10x PCR buffer, 2 mM dNTPs, 25 mM MgSO₄ and KOD-Plus-Neo DNA polymerase) (TOYOBO, catalog number: KOD-401)
9. Agarose (VWR, catalog number: 0710-500g)
10. Quick-Load Purple 1 kb Plus DNA ladder (0.1-10.0 kb) (New England BioLabs, catalog number N0550S)
11. Ethidium bromide (Wako Pure Chemical, catalog number: 547-00101)
12. RNase-free water
13. 4 M ammonium acetate diluted with RNase-free water
14. 99.5% ethanol (Wako Pure Chemical, special grade, catalog number: 057-00451)

15. In Vitro Transcription/Translation Kit (NUProtein, catalog number: PSS3050)
16. Polyacrylamide gel
17. AlphaScreen FLAG® (M2) Detection kit (PerkinElmer, catalog number: 6760613C)
18. AlphaPlate-384, 384-well plate (PerkinElmer, catalog number: 6005350)
19. Tween® 20 (TCI, catalog number: T0543)
20. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: A2058-5G)
21. Polydeoxyadenylic acid-Polythymidylic acid (dAdT) (Sigma-Aldrich, catalog number: P9764-5UN)
22. DL-dithiothreitol (DTT) (Wako Pure Chemical, catalog number: 047-08973)
23. Tris (hydroxymethyl) aminomethane (Wako Pure Chemical, catalog number: 207-06275)
24. Glacial acetic acid (Wako Pure Chemical, catalog number: 518-33985)
25. Ethylenediaminetetraacetic acid (EDTA) (DOJINDO, catalog number: 345-01865)
26. Glycerol (Wako Pure Chemical, catalog number: 075-00616)
27. Sodium dodecyl sulfate (SDS) (Wako Pure Chemical, catalog number: 191-07145)
28. Bromophenol blue (Wako Pure Chemical, catalog number: 021-02911)
29. Xylene cyanolIFF (Wako Pure Chemical, catalog number: 244-00461)
30. 100x TAE buffer (see Recipes)
31. 10x loading dye (see Recipes)

For SDS-PAGE and Western blotting

1. Nitrocellulose membrane (GE Healthcare, catalog number: 10600007)
2. Whatman™ 3 MM Chr Chromatography Paper (GE Healthcare, catalog number: 05-714-5)
3. Hybridization bag
4. MXJB III Film (IBI, catalog number: 6567291)
5. Precision Plus Protein™ Dual color standards (Bio-Rad Laboratories, catalog number: 1610374)
6. SDS polyacrylamide gel
7. Methanol (Wako pure chemical, special grade, catalog number: 131-01826)
8. Anti-DYKDDDDK tag antibody (diluted 1:2,000 in blocking buffer) (Wako Pure Chemical, catalog number: 014-22383)
9. Goat anti-mouse IgG-HRP (diluted 1:1,000 in blocking buffer) (Cosmo Bio, catalog number: 1030-05)
10. SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, catalog number: 34577)
11. Tris (hydroxymethyl) aminomethane (Wako Pure Chemical, catalog number: 207-06275)
12. SDS (Wako Pure Chemical, catalog number: 191-07145)
13. Bromophenol blue (Wako Pure Chemical, catalog number: 021-02911)
14. Xylene cyano IFF (Wako Pure Chemical, catalog number: 244-00461)
15. NaCl (Wako Pure Chemical, catalog number: 191-01665)
16. Na₂HPO₄ (Wako Pure Chemical, catalog number: 197-02865)

17. KCl (Wako Pure Chemical, catalog number: 163-03545)
18. KH₂PO₄ (Wako Pure Chemical, catalog number: 169-04245)
19. Skimmed milk powder (Wako Pure Chemical, catalog number: 190-12865)
20. 10x running buffer (see Recipes)
21. 4x SDS sample buffer (see Recipes)
22. Transfer buffer (see Recipes)
23. 10x PBS (see Recipes)
24. Blocking buffer (see Recipes)

Equipment

1. Pipettes
2. PCR thermal cycler (Thermo Fisher Scientific, model: Veriti 200)
3. Refrigerated centrifuge (Hitachi, model: himac CF 15R)
4. Vortex mixer
5. Block incubator (You can use a water incubator instead of block incubator.)
6. Wonder Shaker (NISSIN, model: NA-4X)
7. Multi Shaker (Tokyo Rikakikai Co., Ltd., model: MMS-120H)
8. Heat Sealer (Taiyo Electric Co., Ltd., model: HS-400)
9. Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories, catalog number: 170-3940)
10. Fuji Medical Film Processor (FUJIFILM Medical, model: FPM100)
11. X-ray film cassette
12. EnSpire™ Alpha Plate Reader (PerkinElmer, model: 2390-00000)

Note: Equipment #5 to #11 are shown for your reference for SDS-PAGE and Western blotting.

Procedure

A. *In vitro* transcription/translation of a transcription factor using AlphaScreen®

1. Preparation of DNA template for *in vitro* transcription/translation

To attach a protein tag, in this case, “FLAG®-tag” to N-terminus of a target transcription factor, perform the two-step PCR method. All primers for making transcription templates are shown in Table 1.

*Note: For details, please refer to articles published in Genes Cells (Nomoto and Tada, 2018a) and Methods Mol Biol (Nomoto and Tada, 2018b) which include all the procedures for *in vitro* transcription/translation.*

Table 1. Primer sequences for transcription template PCR

1st-gene-specific-NF	CCAGCAGGGAGGTACT + target gene-specific 18 bp sequence of
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	5' end with start codon
1st-gene-specific-NR	CCTTATGGCCGGATCCAAGAGCTTTTTTTTTA + target gene-specific 15 bp sequence of 3' end without stop codon
2nd-T7-NF2	CCCGCGAAATTAAATACGACTCACTATAG
2nd-protein tag(FLAG)-NF1	CGACTCACTATAAGGGCTCACCTATCTCTACACAAAACATTCC CTACATACAACTTCAACTTCCTATTATGCATCATCATCATCA TCTCCAGCAGGGAGGTACTATG
2nd-3' SU-NCR2	GGCCCCCCCCTCGAAGG
2nd-3' SU-NR1	CCCTCGAAGGATCAGGCCCTATGCCGGATCAA

- a. Prepare the following 1st PCR mix (total volume: 50 µl/sample) on ice:

5 µl of 10x PCR buffer for KOD-Plus-Neo

5 µl of 2 mM dNTPs

3 µl of 25 mM MgSO₄

1 µl of KOD-Plus-Neo

1 µl of plasmid (1 ng/µl)

1 µl of 10 µM 1st-gene-specific-NF primer

1 µl of 10 µM 1st-gene-specific-NR primer

Add 33 µl of ultrapure water to make up the volume of the reaction to 50 µl

Note: You can use any plasmid containing cDNA region of the gene of interest. In this case, cDNA region cloned into pENTR/D-TOPO was used as a 1st PCR template.

- b. Run the following 1st PCR

Step 1: 94 °C for 5 min

Step 2: 30 cycles of

a) 98 °C for 10 s

b) 55 °C for 30 s

c) 68 °C for 3 min (30-60 s/kb)

Step 3: 72 °C for 2 min

Step 4: 4 °C until agarose gel electrophoresis

Note: Temperature in this step (hybridization step) depends on primer sequences. You can change the temperature for this step according to normal PCR methods. It should be between 55 °C and 65 °C.

- c. Run 2 µl of the 1st PCR sample on agarose gel (1.5% [w/v] in 1x TAE buffer) electrophoresis to check the quantity and size of PCR amplicon (Figure 1).

Note: If multiple PCR amplicons are detected in single PCR, gel extraction of a target band is recommended.

- d. Prepare the following 2nd PCR mix (total volume: 50 µl/sample) on ice:

5 µl of 10x PCR buffer for KOD-Plus-Neo

- 5 µl of 2 mM dNTPs
3 µl of 25 mM MgSO₄
1 µl of KOD-Plus-Neo
1 µl of 1st PCR sample
1 µl of 10 µM 2nd-T7-NF2 primer
1 µl of 100 nM 2nd-protein tag(FLAG)-NF1 primer
1 µl of 10 µM 2nd-3' SU-NCR2 primer
1 µl of 100 nM 2nd-3' SU-NR1 primer
Add 31 µl of ultrapure water to make up the volume of the reaction to 50 µl
- e. Run the following 2nd PCR
- Step 1: 98 °C for 1 min
Step 2: 10 cycles of
a) 98 °C for 10 s
b) 60 °C for 1 min
c) 68 °C for 3 min (30-60 s/kb)
Step 3: 30 cycles of
a) 98 °C for 10 s
b) 60 °C for 15 s
c) 68 °C for 3 min (30-60 s/kb)
Step 4: 72 °C for 2 min
Step 5: 4 °C until agarose gel electrophoresis
- f. Run 2 µl of the 2nd PCR sample on agarose gel (1.5% [w/v] in 1x TAE buffer) electrophoresis to check the quantity and size of PCR amplicon (Figure 1).
- Note: If multiple PCR amplicons are detected in single PCR, gel extraction of a target band is recommended. One transcription reaction requires 25 µl of purified 2nd PCR amplicon containing 300 to 600 ng DNA.*

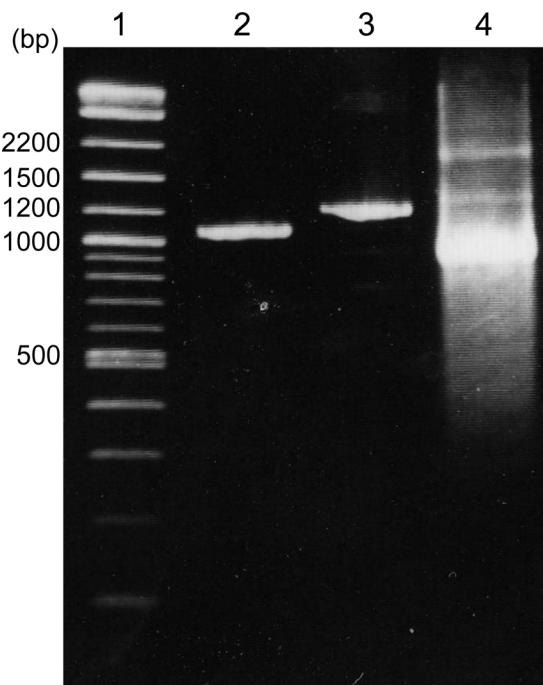


Figure 1. Agarose gel electrophoresis of products from the 1st PCR, 2nd PCR, and transcription products. Lane 1: DNA marker (2-log ladder), lane 2: 1st PCR product, lane 3: 2nd PCR product, lane 4: transcription reaction product. *MYB30* coding region cloned into pENTR/D-TOPO vector is used as the template for 1st PCR. *MYB30* coding region is 969 base pairs.

2. *In vitro* transcription

Perform *In vitro* transcription/translation reaction using NUProtein kit following the manufacturer's protocol.

a. Prepare the following transcriptional master mix (for one sample) on ice:

2.5 µl of 10x Transcription Buffer (provided in the kit)

1.25 µl of 0.1 M DTT (provided in the kit)

2.5 µl of 25 mM NTPs (provided in the kit)

1 µl of T7 RNA polymerase (provided in the kit)

Add 15.25 µl of RNase-free water

Note: If RNA degradation occurs, an RNase inhibitor should be added to the transcription mix.

b. Dispense 22.5 µl of the above transcription mix to each new tube (RNase-free tube recommended) and add 2.5 µl of 2nd PCR product carefully (Purification of 2nd PCR products is not necessarily required).

Note: NUProtein kit contains positive control DNA (2nd PCR amplicon) which can be used as a positive control for the in vitro transcription reaction.

c. Incubate the sample at 37 °C for 3 h in a block incubator.

Note: Incubation time depends on the type and length of mRNA, and may vary from 30 min

to 3 h. Water incubator can also be used instead of block incubator.

- d. Run 1 µl of the transcription reaction sample can be loaded on agarose gel (1.5% [w/v] in 1x TAE buffer) electrophoresis to check the quantity and quality of mRNA (Figure 1).
 - e. Add 10 µl of 4 M ammonium acetate and 100 µl of 99.5 % ethanol per sample (total volume: 124 µl/sample).
 - f. Mix the sample by gentle vortexing or tapping and incubate at -20 °C for 20 min after centrifugation for a few seconds.
 - g. Centrifuge the sample at 17,700 x g for 20 min at 4 °C and discard the supernatant carefully.
 - h. Repeat centrifugation for few seconds to remove the residual solution completely using a pipette.
 - i. Dry the pellet by keeping the tube lid open for about 10 min.
 - j. Add 70 µl of RNase-free water per sample and incubate at RT for about 15 min.
 - k. Dissolve the pellet well by gentle tapping and centrifuging for a few seconds.
3. *In vitro* protein synthesis
 - a. Prepare the following translation reaction master mix (for one sample) into 1.5 ml tube on ice:
 - 20 µl of wheat germ extract (WGE) (provided in the kit)
 - 20 µl of amino acid mixture (AAM) (provided in the kit)
 - b. Mix the translation reaction master mix gently, and incubate at RT for 15 min.
Note: Preincubation of WGE and AAM may enhance the translation efficiency.
 - c. Add 40 µl of the translation reaction mix to 70 µl of prepared mRNA carefully (total volume: 110 µl/sample).
Note: Do not make bubbles during additions. Make AlphaScreen® negative control by adding 40 µl of the translation reaction mix to 70 µl of RNase-free water.
 - d. Incubate the sample at 16 °C for 10 h without agitation.
 - e. Centrifuge the sample at 17,700 x g for 10 min at 4 °C and transfer the supernatant (approximately 118 µl) to a new tube.
 - f. Mix 1 µl of the *in vitro*-synthesized protein, 4 µl of 4x SDS sample buffer, 1 µl of 2 M DTT, and 6 µl of water in a new tube.
Note: Remaining protein sample can be stored at -80 °C. We recommend flash-freezing the sample using liquid N₂. Repeated freeze-thaw cycles decrease the activity of synthesized proteins. When using frozen protein for any assay, quickly thaw the sample by hand.
 - g. Heat protein sample at 70 °C for 20 min and perform SDS-PAGE in a 10% (w/v) polyacrylamide gel and confirm the synthesized target protein by western blotting with anti-DYKDDDDK tag antibody.

B. AlphaScreen® assay

1. Preparation of double-stranded DNA probes by annealing

For AlphaScreen®, detecting the interaction between protein and DNA, prepare biotinylated (target sample) and non-biotinylated (control for the detection) 50 bp DNA probes. For obtaining stable AlphaScreen® results, we recommend designing the DNA probes that contain putative DNA binding sequence of the target transcription factor in the central region (11 bp to 40 bp from 5' end of the probe [Figure 2A]).

- Mix 20 μ l of 20 μ M biotinylated 50 bp single-strand DNA (or 20 μ l of 20 μ M non-biotinylated 50 bp single strand DNA) and 20 μ l of 20 μ M complementary unmodified 50 bp single-strand DNA in 1.5 ml tube (total volume: 40 μ l).

- Incubate the DNA mixture at 95 °C for 10 min, let it cool down until it reaches RT.

Note: Double-strand DNA probes can be stored at -20 °C.

- Check annealed double-stranded DNA probes by polyacrylamide gel (12% w/v) electrophoresis (Figure 2B).

Note: At the same time prepare DNA probe for the negative control, which does not contain any putative DNA binding sequence of the target transcription factor. If required, also prepare the DNA probes that contain mutation in the target sequence.

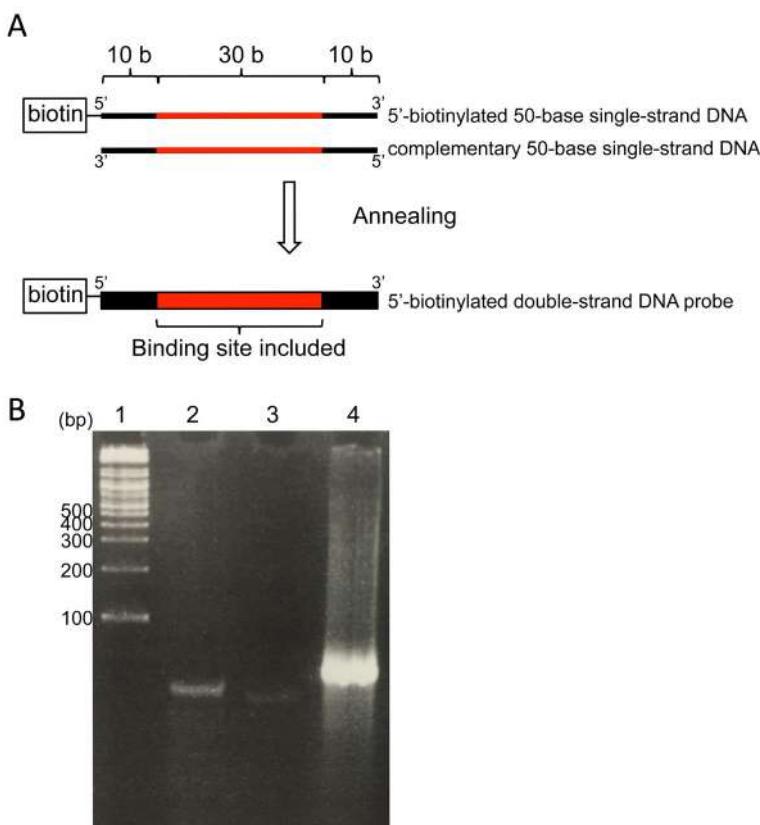


Figure 2. Schematic diagram of DNA probe for AlphaScreen®. A. Schematic diagram of annealed double-strand DNA probe. Black lines and box indicate scaffold region (10 bases in length). Red lines and box indicate the region that contains putative DNA binding sequence of the target transcription factor. B. Polyacrylamide gel (10%) electrophoresis of DNA probes. Lane 1: DNA marker (2-log ladder), lane 2: 5'-biotinylated 50-base single-strand DNA, lane 3:

Complementary 50-base single-strand DNA, lane 4: Annealed double-strand DNA. The polyacrylamide gel was stained with ethidium bromide after electrophoresis.

2. AlphaScreen® assay with FLAG (M2) Detection kit

- a. Prepare the following protein-DNA binding mix (total volume: 13 µl/sample):

2.5 µl of 10x control buffer [provided in FLAG (M2) Detection kit]

2.5 µl of 0.1% (w/v) Tween® 20

2.5 µl of 1% (w/v) BSA

1 µl of 1 ng/µl dAdT

Add 4.5 µl of ultrapure water.

Note: Depending on the target transcription factor, dGdC should be used instead of dAdT.

- b. Dispense 13.0 µl of the protein-DNA binding mix to the 384-well plate and add 2 µl of 625 nM of double-stranded DNA probe and 4-fold diluted FLAG-tagged target transcription factor protein.

Note: Also add 2 µl of 4-fold diluted negative control protein.

- c. Incubate protein-DNA binding mix at RT for 1 h.

Note: Dilution range of synthesized protein depends on the feature of target transcription factor. Two to ten times dilution can be used for this assay. Incubation under dark condition is not necessary for this step.

- d. Add 4 µl of 40-fold diluted (with ultrapure water) acceptor beads and incubate at RT for 1 h.

Note: Incubation under dark condition is not necessary for this step.

- e. Add 4 µl of 40-fold diluted (with ultrapure water) donor beads and incubate at RT for 1 h to 12 h in the dark (total volume: 25 µl).

Note: To ensure that the plate is in the dark, wrap it with aluminum foil and remove it before keeping it in the Alpha plate reader.

- f. Detect signal by EnSpire™ Alpha Plate Reader. After the excitation at 680 nm, the emission wavelengths between 520 and 620 nm are measured as AlphaScreen® unit.

Data analysis

1. Divide the signal intensity from the mix of biotinylated DNA probe by the signal intensity from the mix of non-biotinylated DNA probe. This signal can be used as “Relative signal intensity” for plotting a graph.

Note: The signal intensity from negative controls (without target protein) usually shows very low signal.

2. Data from 3 technical replicates are combined for the statistical analysis, and the same experiment using different batches of proteins is repeated at least 3 times with similar results.

Note: One example of AlphaScreen® result can be found in Figures 4B and 4C in Mabuchi et al., 2018. As an alternative way of representing data, you may also be able to show all the

signals, including one from negative controls.

Notes

1. In this protocol, we used protein that is synthesized by our *in vitro* transcription/translation system. However, one may use protein that is synthesized by other lab protocols (e.g., using bacteria, yeast, and any other systems).
2. In this protocol, we use FLAG®-tagged protein. However, one may use other protein tags, (e.g., GST, 6-Histidine, c-myc, and HA) as long as detection kits for “AlphaScreen® Fusion Tag” are available from PerkinElmer (see also web site: www.perkinelmer.com/alphascreen).

Recipes

1. 100x TAE buffer
400 mM Tris-HCl
400 mM glacial acetic acid
10 mM EDTA, pH 8.0
2. 10x loading dye
50% (w/v) glycerol
0.9% (w/v) SDS
10 mM EDTA, pH 8.0
0.05% (w/v) bromophenol blue
0.05% (w/v) xylene cyanol
3. 4x SDS sample buffer
200 mM Tris-HCl, pH 6.8
40% (w/v) glycerol
8% (w/v) SDS
0.08% (w/v) bromophenol blue
4. 10x running buffer
250 mM Tris
1.92 M glycine
1% (w/v) SDS
5. Transfer buffer
192 mM glycine
20% (w/v) methanol
25 mM Tris
6. 10x PBS
1.37 M NaCl

81 mM Na₂HPO₄

26.8 mM KCl

14.7 mM KH₂PO₄

7. Blocking buffer

1x PBS

1% (w/v) non-fat skimmed milk

0.1% (w/v) Tween® 20

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Competing interests

The authors declare no conflicts of interest or competing interests.

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Quantification of Serotonin in Rice and Insect Pest and its Functional Analysis in Insects Using Artificial Diet Feeding

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[Abstract] Rice is one of the world's most important crops, but its production suffers from insect pests. Rice brown planthopper (BPH; *Nilaparvata lugens* Stål) and striped stem borer (SSB, *Chilo suppressalis* Walker) are the two most serious pests in rice production. We reported that serotonin is an essential mediator in the interaction between rice and insect. Here, we established a method for extraction and determination of serotonin in rice and BPH.

Keywords: Serotonin, Rice, Artificial diet, BPH, SSB, Insect resistance

[Background] Serotonin (5-hydroxytryptamine) is an important signaling molecule in animals. It has also been shown to be distributed widely in plants. In our previous research, we reported that deficiency of serotonin in rice plants enhances its BPH and SSB resistance, and the addition of serotonin in plant growth medium results in a loss of insect resistance in mutants (Lu et al., 2018). Here, we provide detailed protocols for determination of serotonin in rice plants, and in SSB and BPH insects, together with the methods for feeding SSB and BPH in artificial diets (added with exogenous serotonin).

Materials and Reagents

A. Serotonin measurement

1. Sep-Pak C18 cartridge (Waters, catalog number: WAT020515)
2. 0.20 µm filter (Millex-LG Millipore, catalog number: SLLGH13NL)
3. Syringe (10 ml, Aladdin, A2292-05)
4. Syringe (1 ml, Aladdin, A2292-01)
5. 50 ml tubes
6. 2 ml centrifuge tubes
7. 1.5 ml centrifuge tubes
8. Rice (*Oryza sativa* L.) plants, grown in indoor facilities or in the field

Note: In this experiment, the wild type (WT) cultivar Jiazhe B and its OsT5H (encoding Tryptamine 5 hydrolyase) mutant line Jiazhe LM (Lu et al., 2016) were used.

9. Brown planthopper (BPH; *Nilaparvata lugens* Stål), raised in laboratory by feeding on one-month-old TN1 (a susceptible rice variety) plants (originally collected from the field)
10. Striped stem borer (SSB, *Chilo suppressalis* Walker), raised in laboratory by feeding on artificial

diet

11. Liquid Nitrogen
12. Methanol (Aladdin, catalog number: M117118)
13. Trifluoroacetic acid (Aladdin, catalog number: T103297)
14. Perchloric acid (Aladdin, catalog number: P112070)
15. Serotonin standard (Sigma, catalog number: 14927)
16. Ammonium acetate
17. Formic acid
18. Nitrogen
19. Argon

B. Assay of BPH survival rate by artificial diet feeding

1. Glass cylinder (Figures 2A-2B), home-made, ø, 4 cm; height, 8 cm; with 48 small holes (ø, 0.8 mm)
2. Parafilm M (Parafilm, PM996)
3. 1.5 ml tubes (centrifuge tubes)
4. Filter paper (Sangon Biotech, catalog number: F503316)
5. Plastic tube (24 x 95 mm)
6. Glycine (Sangon Biotech, catalog number: A100167)
7. L-Alanine (Sangon Biotech, catalog number: A600022)
8. L-Arginine hydrochloride (Sangon Biotech, catalog number: A600205)
9. L-Asparagine (Sangon Biotech, catalog number: A694341)
10. L-Aspartic acid (Sangon Biotech, catalog number: A600091)
11. L-Cysteine (Sangon Biotech, catalog number: A600132)
12. γ-Amino butyric acid (Sangon Biotech, catalog number: A600040)
13. L-Glutamic acid (Sangon Biotech, catalog number: A600221)
14. L-Glutamine (Sangon Biotech, catalog number: A100374)
15. L-Histidine (Sangon Biotech, catalog number: A604351)
16. L-Methionine (Sangon Biotech, catalog number: A100801)
17. L-Isoleucine (Sangon Biotech, catalog number: A100803)
18. L-Leucine (Sangon Biotech, catalog number: A100811)
19. L-Proline (Sangon Biotech, catalog number: A600923)
20. L-Lysine hydrochloride (Sangon Biotech, catalog number: A110437)
21. L-Phenylalanine (Sangon Biotech, catalog number: A600991)
22. L-Serine (Sangon Biotech, catalog number: A601479)
23. L-Threonine (Sangon Biotech, catalog number: A610919)
24. L-Tryptophan (Sangon Biotech, catalog number: A601911)
25. L-Tyrosine (Sangon Biotech, catalog number: A601932)
26. L-Valine (Sangon Biotech, catalog number: A600172)

27. Biotin (Sangon Biotech, catalog number: A100340)
 28. Calcium pantothenate (Sangon Biotech, catalog number: A600683)
 29. Choline chloride (Sangon Biotech, catalog number: A600299)
 30. Folic acid (Sangon Biotech, catalog number: A610466)
 31. Inositol (Sangon Biotech, catalog number: A600536)
 32. Nicotinic acid (Sangon Biotech, catalog number: A610660)
 33. Pyridoxine hydrochloride (Sangon Biotech, catalog number: A600797)
 34. Riboflavin (Sangon Biotech, catalog number: A600470)
 35. Thiamine hydrochloride (Sangon Biotech, catalog number: A500986)
 36. Ascorbic acid (Sangon Biotech, catalog number: A100143)
 37. CaCl₂·2H₂O (Sangon Biotech, catalog number: A100556)
 38. CuCl₂·2H₂O (Sangon Biotech, catalog number: A603090)
 39. FeCl₃·6H₂O (Sangon Biotech, catalog number: A600201)
 40. MnCl₂·4H₂O (Sangon Biotech, catalog number: A500331)
 41. ZnCl₂ (Sangon Biotech, catalog number: A501003)
 42. MgCl₂·6H₂O (Sangon Biotech, catalog number: A100288)
 43. KH₂PO₄ (Sangon Biotech, catalog number: A100781)
 44. Sucrose (Sangon Biotech, catalog number: A100335)
 45. BPH artificial diet (D-97) (see Recipes)
- C. Assay of SSB body weight by artificial diet feeding
1. Plastic barrel
 2. Fresh wild rice (*Zizania aquatica*) stem (From supermarket) (Han et al., 2012)
 3. Soybean flour (From supermarket, a China brand)
 4. Yeast extract powder (From supermarket, a China brand)
 5. Casein (Sigma, catalog number: C7078)
 6. Sucrose (Sinopharm Chemical Reagent Co., Ltd., catalog number: A100335)
 7. Ascorbic acid (Sangon Biotech, catalog number: A100143)
 8. Sorbic acid (Sangon Biotech, catalog number: SB0899)
 9. Cholesterol (Sangon Biotech, catalog number: C0433)
 10. Choline chloride (AMRESCO, catalog number: 0448)
 11. Wesson's salt Mixture (Sigma, catalog number: W1374)
 12. 40% Formaldehyde (Sinopharm Chemical Reagent Co., Ltd., Catalog number: 10010018)
 13. Methyl parahydroxybenzoats
 14. Agar (Sinopharm Chemical Reagent Co., Ltd., 10000561)
 15. Nicotinic acid amide (Sangon Biotech, catalog number: A510659)
 16. Pyridoxine hydrochloride (Sangon Biotech, catalog number: A600797)
 17. Riboflavin (Sangon Biotech, catalog number: A600470)
 18. Thiamine hydrochloride (Sangon Biotech, catalog number: A500986)

19. Cyanocobalamin (Fluka, catalog number: 82897)
20. Folic acid (Sangon Biotech, catalog number: A610466)
21. Calcium pantothenate (Sangon Biotech, catalog number: A600683)
22. Biotin (Sangon Biotech, catalog number: A100340)
23. Semi-artificial diets recipe for SSB (see Recipes)

Equipment

1. Refrigerated shaker (Hualida Co., Ltd., model: HZ-9210K)
2. Centrifuge (Eppendorf, model: 5804R)
3. Rotary evaporator (Shanghai Yarong Biochemical instrument Co., Ltd., model: RE52CS)
4. -20 °C freezer
5. High performance liquid chromatography, Agilent 1200 infinity series with diode array detector (DAD) in plant serotonin measurement
6. HPLC-MS system, Agilent Technologies 1290 with Agilent 6460 triple mass selective detector under multi reaction monitoring (MRM) working mode in BPH serotonin measurement
7. C18 column (Agilent, TC-C18(2), 4.6 x 250 mm, 5 µm) for HPLC
8. C18 column (Agilent, Zorbax XDB, 2.1 x 150 mm, 3.5 µm) for HPLC-MS
9. Analytical balance with 0.00001 g accuracy
10. -80 °C freezer
11. 4 °C refrigerator
12. Growth chamber
13. Blender
14. Autoclave
15. Crisper

Software

1. Variance (ANOVA) program StaView, used for statistical analyses

Procedure

A. Quantification of serotonin levels in plants

Levels of serotonin in plants were quantified by HPLC according to Kang *et al.* (2007) with some modifications.

1. Grind fresh tissue (of leaf/stem/root) in liquid nitrogen using a pestle and immediately store in a -80 °C freezer.
2. Weight one to three grams powder and dissolve in 8 ml methanol by shaking for 10 min on an orbital shaker (4 °C, 200 rpm).

3. Centrifuge the mixed solution at 13,500 $\times g$ for 5 min at 4 °C.
4. Transfer and filter the supernatant to a new tube using a syringe with a Millex-LG filter (0.2 μm), Add 2 ml distilled water (1/4 volume of the total) at room temperature.
5. Slowly inject the above solution into a pre-activated Sep-pak C18 cartridge (first wash with methanol in three times volume of the cartridge column, followed by distilled water of the same amount) and collect effluent into a 50 ml tube.
6. Wash the cartridge using 10 ml 80% methanol and collect the effluent into the 50 ml tube.
7. Transfer the effluent into a round-bottomed flask for drying in a rotary evaporator (40 °C under vacuum).
8. Add 500 μl 50% methanol into the flask and dissolve the residue by slightly shaking.
9. Analyze the serotonin level on reverse-phase HPLC. Inject 10 μl sample solution into a C18 column, mixed with an isocratic solution of 10% methanol in water containing 0.3% trifluoroacetic acid, at a flow rate of 0.8 ml/min, and detect the signal at 280 nm.
10. Prepare standard serotonin at concentrations of 2, 1, 0.5, 0.2, 0.1 $\mu\text{g/ml}$. Analyze 10 μl of serotonin standard solution as above to determine the retention time and quantitative relationship between concentration and peak area.
11. Calculate the serotonin concentration according to serotonin retention time point.

B. Quantification of serotonin levels in brown planthopper (BPH) (Figure 1)

The content of serotonin in BPH body is lower compared with that in plants, hence the measurement of serotonin needs to be performed on HPLC-MS, here is the protocol modified from Ma et al. (2011).

1. Collect about 15 adult insects from rice plants into 2 ml centrifuge tubes. After starving for 12 h, freeze in liquid nitrogen, homogenize into powder and store in a -80 °C freezer.
2. Transfer 10-20 mg insect homogenate to 1.5 ml centrifuge tubes, add 300 μl ice-cold 0.1 M perchloric acid, after brief vortexing, and keep on ice for 10 min.
3. Centrifuge the mixture at 14,000 $\times g$ for 10 min at 4 °C.
4. Transfer and filter the supernatant to a new tube using a syringe with a Millex-LG filter (0.2 μm), and store at -20 °C until HPLC-MS analysis.
5. Analyze the serotonin level on HPLC-MS system, Agilent Technologies 1290 with Agilent 6460 triple mass selective detector under multi reaction monitoring (MRM) working mode. Inject 10 μl sample solution into a C18 column (Zorbax XDB, 2.1 x 150 mm, 3.5 μm). Mix with a gradient elution at a flow rate of 0.3 ml/min. The elution is composed of 2 mmol/L ammonium acetate containing 0.1% formic acid and methanol with increasing methanol proportion from 19% to 90% (v/v) during the first 2.5 min, and remain the methanol proportion at 90% from 2.5 to 4.5 min.
6. Settings for the MS system are: gas temperature: 325 °C, gas flow: 5 L/min, sheath gas temperature: 350 °C, sheath gas flow: 11 L/min, nebulizer pressure: 344.8 kPa, capillary voltage: 3,000 V, nozzle voltage: 500 V in positive mode. Parent ion (m/z) 177, daughter ion (m/z) 160. Use nitrogen as the drying and sheath gas, and argon as collision gas.
7. Prepare standard serotonin solution at concentrations of 2, 1, 0.5 ng/ml. Analyze 10 μl of

serotonin standard solution as above.

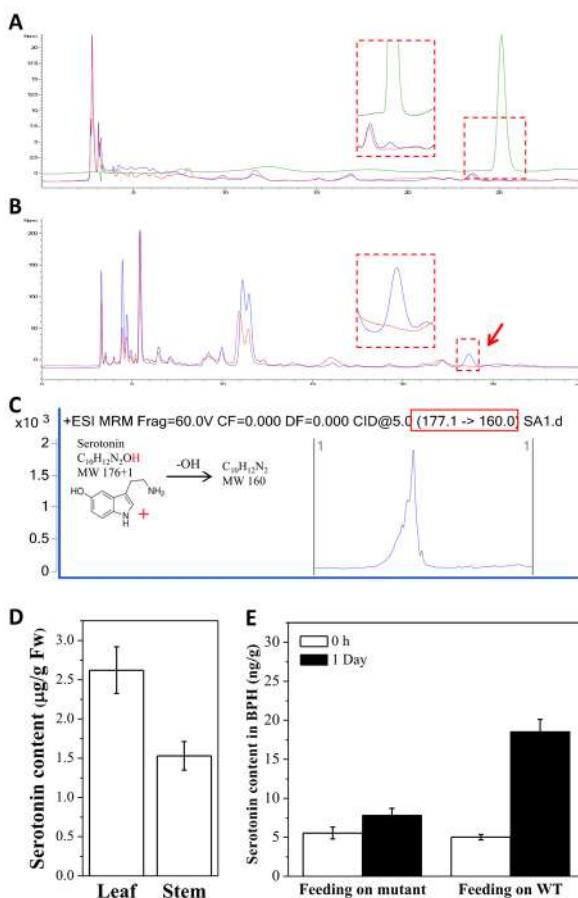


Figure 1. Chromatogram of serotonin measurement (A-C) and serotonin content in rice tissues (D) and in BPH female adults feeding on either WT or mutant rice lines. A. HPLC chromatogram of serotonin, standard (green line), the WT (Jiazhe B, blue line) and its *OsT5H* mutant. B. HPLC chromatogram of serotonin in WT (blue line) and the mutant (red line). The peak pointed by the red arrow means that the compound serotonin is deficient in the mutants. C. Serotonin measurement using HPLC-MS in multi reaction monitoring (MRM) working mode. D. Serotonin content in leaf and stem of WT Jiazhe B. E. Serotonin content in BPH females after feeding on WT (Jiazhe B) or mutant (Jiazhe LM) rice plants. Prior to the assay, female adults were starved for 12 h.

C. BPH artificial diet experiment

The artificial diet experiment was performed as previously described (Fu et al., 2001; Ji et al., 2017).

1. Prepare artificial diet (D-97) for BPH according to Koyama et al. (1988), modified by Fu et al. (2001), supplemented with serotonin at concentrations of 0.1 and 1 μ g/ml.
2. Cover one end of the glass cylinder (Figures 2A-2B) with parafilm, drop 40 μ l artificial diet (Figure 2C) on to the parafilm at the center, cover with another layer of parafilm (Figures 2D-2F).

3. Insert a piece of wet filter paper (2 x 6 cm) and release 20 second-instar BPH nymphs into the cylinder (Figure 2G).
4. Cover the other end as Step C2. Wrap the cylinder with a black cotton cloth (Figure 2H).
5. Place the cylinders in a growth chamber at 28 °C under 16 h/8 h day/night cycles with a photon flux density of 250 μmol of photons $\text{m}^{-2} \text{s}^{-1}$.
6. Every day, transfer survived nymphs to a new cylinder (prepared the same as above) with fresh diets, and record the mortality at the same time for five days.

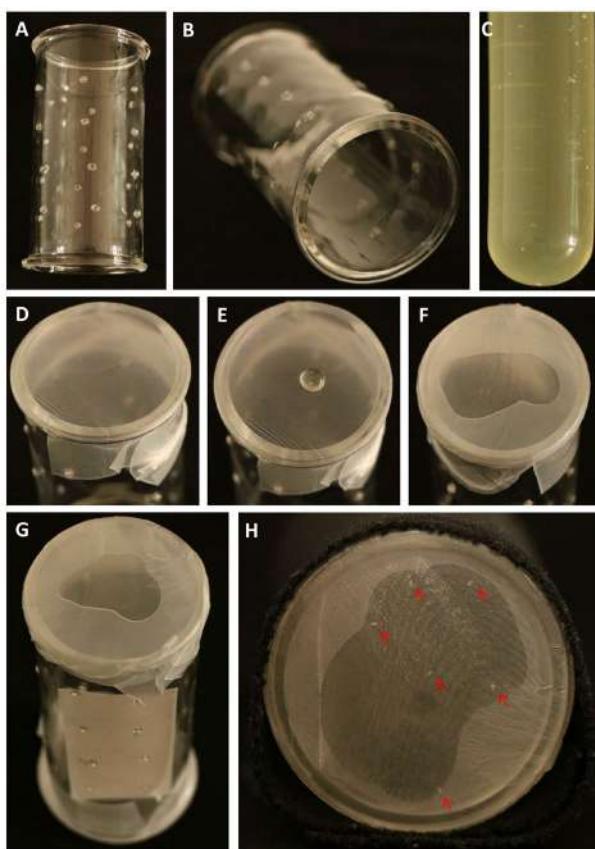


Figure 2. Step-by-step procedure for preparation of cylinders for BPH artificial diet experiment

D. SSB artificial diet experiment

1. Prepare an artificial diet according to Han et al. (2012), with supplementation of 1 $\mu\text{g/g}$ serotonin as appropriate.
2. Cut artificial diet into small patches (Figure 3A) and insert one into each plastic tube (24 x 95 mm).
3. For production of neonates, thread 2-3 leaf segments with SSB eggs in one insect pin and place in the tube with artificial diets without serotonin supplementation (Figure 3B).
4. Transfer one neonate into one tube with one pellet of the artificial diet supplemented with 1 $\mu\text{g/g}$ serotonin (Figure 3C).

5. Place the tubes in a growth chamber with a photon flux density of 250 μM of photos $\text{m}^{-2} \text{s}^{-1}$ in an 8 h light/16 h dark regime at 28 °C. Replace diet every two days (Figure 3D)
6. Examine the larvae development stage every day, and weight larvae after each molting until the 6th instar stage.

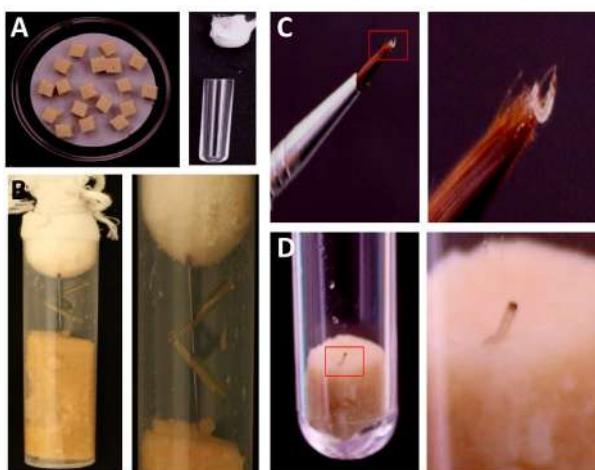


Figure 3. Step-by-step procedure for SSB egg hatching and artificial diet experiment

In support of the role of serotonin in BPH and SSB resistance in rice, the beneficial effect of this hormone was demonstrated in artificial diets, with increased performance of both BPH (Figure 4A) and SSB (Figure 4B).

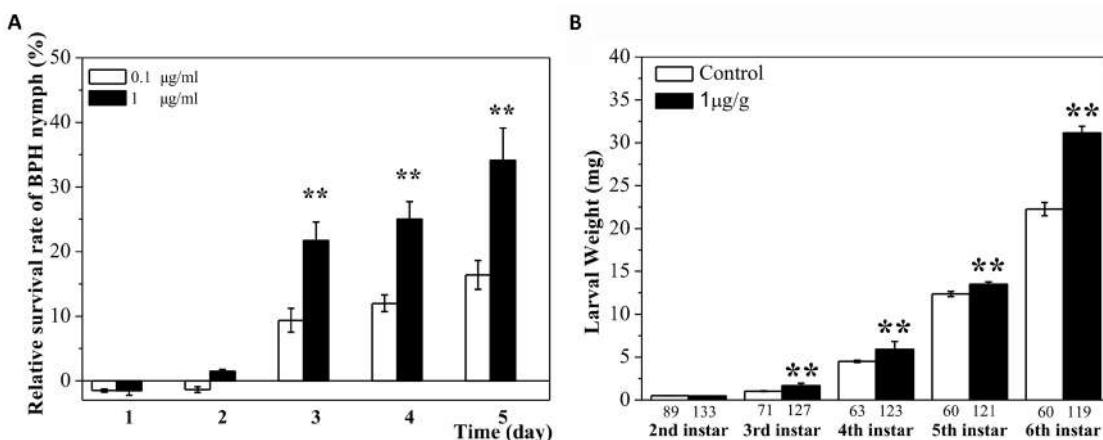


Figure 4. Addition of exogenous serotonin attracts BPH and SSB feeding. A. Addition of serotonin to the artificial diet increases BPH survival rates. Relative survival rate (%) = $(N_t - N_c)/N_c \times 100$, N_c and N_t stand for the number of survived nymphs feeding on an artificial diet without and with serotonin, respectively, at a given time point. B. Addition of serotonin in artificial diet enhances SSB performance. Control stands for artificial diet without serotonin. One hundred SSB larvae were used per treatment.

Data analysis

Statistical analyses were performed using the one-way analysis of variance (ANOVA) programme StaView in these experiments. Data are present in mean values with standard errors as error bars. The differences were considered to be significant when the probability (*P*) was less than 0.05 in Tukey test.

Notes

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Recipes

1. BPH artificial diet (D-97)

For preparation of 100 ml diet, add (mg):

Glycine	30
L-Alanine	130
L-Arginine hydrochloride	175
L-Asparagine	230
L-Aspartic acid	100
L-Cysteine	80
L-Cystine hydrochloride	20
γ -Amino butyric acid	10
L-Glutamic acid	250
L-Glutamine	240
L-Histidine	80
L-Methionine	70
L-Isoleucine	100
L-Leucine	240
L-Proline	100
L-Lysine hydrochloride	200
L-Phenylalanine	200
L-Serine	400
L-Threonine	130
L-Tryptophane	105
L-Tyrosine	10
L-Valine	300
Biotin	0.05

Calcium pantothenate	5.0
Choline chloride	50.0
Folic acid	0.5
Inositol	50.0
Nicotinic acid	15.0
Pyridoxine hydrochloride	2.5
Riboflavin	0.5
Thiamine hydrochloride	2.5
Ascorbic acid	100.0
CaCl ₂ ·2H ₂ O	3.115
CuCl ₂ ·2H ₂ O	0.268
FeCl ₃ ·6H ₂ O	2.228
MnCl ₂ ·4H ₂ O	0.793
ZnCl ₂	0.396
MgCl ₂ ·6H ₂ O	200
KH ₂ PO ₄	500
Sucrose	9,000

Add distilled water to a total volume of 100 ml

Adjust the pH to 6.8

2. Semi-artificial diets recipe for SSB (5 L)

- a. Cut 1,000 g fresh *Zizania aquatica* into patches and homogenize in 2,300 ml water in a blender
- b. Mix the homogenate with 300 g soybean flour, 200 g yeast extract powder, 100 g casein and 100 g sucrose in a plastic barrel (10 L). Sterilize the mixture under 121 °C for 30 min in an autoclave
- c. Heat 120 g agar in 2,500 ml water until boiling, pour the above autoclaved mixture into the agar solution
- d. Prepare 200 ml solution with vitamin C 30 g, sorbic acid 10 g, cholesterol 2 g, choline chloride 3 g, Wesson's salt mixture 1 g, methyl parahydroxybenzoats 10 g and vitamin complex 1 package (containing nicotinic acid amide 0.05216 g; vitamin B1: 0.01312 g; vitamin B2: 0.02624 g; vitamin B6: 0.01312 g; vitamin B12: 0.00032 g; folic acid: 0.01312 g; calcium pantothenate: 0.05216 g; biotin: 0.00128 g)
- e. Pour the 200 ml solution into the *Zizania aquatica* mixture when it is cooled down to 60 °C, and add 6 ml 40% formaldehyde
- f. After blended well, put the mixture into crispers and store at 4 °C

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Competing interests

There are no any conflicts of interest or competing interests.

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Top Starch Plating Method for the Efficient Cultivation of Unicellular Red Alga *Cyanidioschyzon merolae*

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[Abstract] The unicellular red alga *Cyanidioschyzon merolae* has been used as a model photosynthetic eukaryote for various basic and applied studies, and several of these molecular genetics techniques have been reported. However, there are still improvements to be made concerning the plating method. The conventional plating method often generates diffuse colonies and single colonies cannot be easily isolated. To overcome these problems, we established a novel plating method for *C. merolae*, making use of melted cornstarch as the use of top agar plating in bacterial genetics. This method improved the formation of defined colonies in at least 4-fold higher efficiency than the conventional method, and made the handling procedure much easier than the previous method.

Keywords: *Cyanidioschyzon merolae*, Plating method, Single colony isolation, Top starch solution method, MA2

[Background] *Cyanidioschyzon merolae* 10D is a unicellular red alga that inhabits sulfate acid hot springs (Matsuzaki *et al.*, 2004). It has a simple cellular structure, composed of a minimum set of organelles: one nucleus, one mitochondrion, and one plastid in a cell. *C. merolae* is used as a model organism to investigate the basic architecture of eukaryotes. Thus, many molecular genetics techniques have been developed using this organism, including antisense suppression, transient expression, gene disruption, and stable insertion into the genome (Minoda *et al.*, 2004; Ohnuma *et al.*, 2008; Imamura *et al.*, 2009; Imamura *et al.*, 2010; Zienkiewicz *et al.*, 2017a; Zienkiewicz *et al.*, 2017b; Zienkiewicz *et al.*, 2018). The plating method for the selection of transformants is important for transformation experiments. However, *C. merolae* shows considerably low plating efficiencies (less than 0.5%) on MA2 solid gellan gum plates. The direct spread of cells onto an MA2 solid gellan gum plate usually results in the death of the cells (Figure 1A). Hence, the plating method has been modified using the cornstarch embedding method which is commonly used for the cell-wall-less *Chlamydomonas reinhardtii* strain (Shimogawara *et al.*, 1998). In the cornstarch embedding method, cells are grown on a starch bed prepared by spotting 20% slurry cornstarch on the MA2 solid gellan gum. Cells form colonies on the solid starch bed, but these are often diffused or blurred (Figure 1B).

To improve these points, we here devised a novel plating method using melted cornstarch (named in this protocol “top starch solution method”), similar to the top-agar plating method used in bacterial genetics. The 2% cornstarch in dH₂O was melted at 98 °C for 10 min. The melted cornstarch solution

(= top starch solution) was mixed with an equal volume of the liquid MA2 medium containing *C. merolae* cells, and poured onto the surface of the MA2 solid gellan gum. Cells formed defined colonies using this plating method (Figure 1C). The plating efficiency of the top starch solution method was more than 2.0%, while that of the conventional method was less than 0.5%. In the conventional method, it was necessary to prepare many starch beds on solid plates prior to use, but the top starch solution method only requires the pouring of the melted starch-cell mixture onto the solid medium, making the plating procedure quick and easy. In this report, we describe the detailed procedure of the top starch solution method.

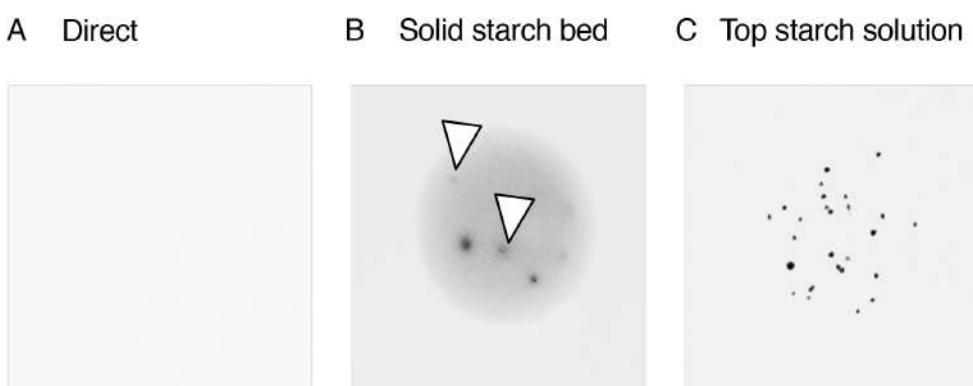


Figure 1. Effect of the top starch solution method on the colony formation in *C. merolae*. (A-C) In each approach, plates with the spotted cells were incubated for two weeks at 40 °C under continuous white light. A. Five hundred cells were directly spotted onto an MA2 solid gellan gum plate, which resulted in no visible colony formation. B. Solid starch beds were made using 15 µl of 20% slurry cornstarch in an MA2 liquid medium. Five hundred cells were spotted onto a solid cornstarch bed. Open triangles indicate diffused, blurred colonies. C. The 2% cornstarch in dH₂O was melted at 98 °C for 10 min to make the top starch solution. The top starch solution was mixed with an equal volume of cells in MA2 medium. Fifteen microliter aliquots containing 500 cells were spotted onto the MA2 solid gellan gum plate.

Materials and Reagents

1. Pipette tips (NIPPON Genetics Co., Ltd., catalog numbers: FG-102, FG-301, FG-401)
2. Parafilm (LMS Co., Ltd., catalog number: PF, PM-996)
3. 1.5 ml microtube (BM Equipment Co., Ltd., catalog number: NT-175)
4. 2.0 ml microtube (WATSON Co., Ltd., catalog number: 132-620C)
5. 50 ml centrifuge tube (Corning Incorporated., catalog number: 352196)
6. AnaeroPouch (Mitsubishi Gas, catalog number: 2-3764-02)
7. AnaeroPack (Mitsubishi Gas, catalog number: 2-3765-01)
8. Sterilized plastic plate (Ø 90 mm) (As One Corporation., catalog number: GD90-15)
9. *Cyanidioschyzon merolae* 10D [available as NIES-3377 from NIES collection, Tsukuba, Japan (http://mcc.nies.go.jp/index_en.html)]

10. Cornstarch (Kawamitsu-Bussan, catalog number: 4901 486 02701 6)
11. Gellan gum (FUJIFILM Wako Pure Chemical Corporation, catalog number: 073-03071)
12. Uracil (Sigma-Aldrich, catalog number: U0750-5G)
13. 5-Fluoroorotic acid monohydrate (5-FOA) (FUJIFILM Wako Pure Chemical Corporation, catalog number: 003234)
14. Ethanol (FUJIFILM Wako Pure Chemical Corporation, catalog number: 055-00457)
15. $(\text{NH}_4)_2\text{SO}_4$ (FUJIFILM Wako Pure Chemical Corporation, catalog number: 019-03435)
16. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (FUJIFILM Wako Pure Chemical Corporation, catalog number: 138-00415)
17. H_2SO_4 (FUJIFILM Wako Pure Chemical Corporation, catalog number: 192-04696)
18. H_3BO_3 (FUJIFILM Wako Pure Chemical Corporation, catalog number: 021-02195)
19. $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (FUJIFILM Wako Pure Chemical Corporation, catalog number: 133-00725)
20. ZnCl_2 (FUJIFILM Wako Pure Chemical Corporation, catalog number: 268-01022)
21. $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (FUJIFILM Wako Pure Chemical Corporation, catalog number: 198-02471)
22. $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (FUJIFILM Wako Pure Chemical Corporation, catalog number: 038-03681)
23. $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (FUJIFILM Wako Pure Chemical Corporation, catalog number: 039-04135)
24. KH_2PO_4 (FUJIFILM Wako Pure Chemical Corporation, catalog number: 169-04245)
25. $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (FUJIFILM Wako Pure Chemical Corporation, catalog number: 038-19735)
26. $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (FUJIFILM Wako Pure Chemical Corporation, catalog number: 090-02802)
27. Na_2EDTA (FUJIFILM Wako Pure Chemical Corporation, catalog number: 345-01865)
28. 20% slurry cornstarch stock solution (see Recipes)
29. MA2 medium (Kobayashi *et al.*, 2010) (see Recipes)
 - a. MA2 solution I
 - b. A6 minor salts
 - c. MA2 solution II
 - d. MA2 solution III
 - e. MA2 solution IV
30. MA2 solid gellan gum plate (see Recipes)

Equipment

1. Pipettes (Gilson, Inc., catalog numbers: F123600, F123601, F123602)
2. Refrigerated centrifuge (Koki Holdings Co., Ltd., model: CF16RXII)
3. Microcentrifuge (TOMY Seiko Co., Ltd., model: MX150)
4. 500 ml flask (IWAI, catalog number: 4442FK500)
5. Heat block (Chiyoda Science Co., Ltd., model: MiniT-100)
6. Bio incubator (TOMY Seiko Co., Ltd., model: CLE-303)
7. Clean bench (Panasonic Healthcare Co., Ltd., model: MCV-131BNF)
8. Vortex mixer (M & S Instruments Inc., model: SI-0286)
9. Sterile filter (\varnothing 0.22 μm , Merck, catalog number: SLGV033RS)

Procedure

1. Prepare the MA2 solid gellan gum plate and the 20% slurry cornstarch stock solution (see Recipes).
2. Mix 100 μ l of the cornstarch slurry stock solution and 900 μ l of sterile dH₂O in a 2 ml microtube.
3. Centrifuge the microtubes at 500 $\times g$ for 1 min at room temperature and discard the supernatant.
4. Add 900 μ l of sterile dH₂O and mix vigorously.
5. Centrifuge again at 500 $\times g$ for 1 min at room temperature and discard the supernatant.
6. Add 900 μ l of sterile dH₂O and mix vigorously.
7. Incubate the microtube at 98 °C for 10 min but continue to mix thoroughly by inverting the tube every 2 min.
8. Cool down at room temperature for 10 min (= top starch solution).
Note: Steps 8-11 should be performed in a laminar flow cabinet.
9. Add 1 ml of MA2 culture medium containing *C. merolae* cells (in case of wild type, 500 cells by microscopy observation) to the top starch solution and mix gently.
10. Pour the mixture onto the surface of the MA2 solid gellan gum plate and spread the mixture over the whole plate area by tilting the plate to spread the top starch solution uniformly.
11. Leave the plate open for 15-20 min to allow the top starch solution to solidify. After this step, the surface of the top starch is still wet, and the plate should be incubated with the top starch solution pointing upwards for the entire time.
12. Incubate the MA2 solid gellan gum plate at 40 °C under continuous white light ($35-50 \mu\text{mol m}^{-2} \text{s}^{-1}$) supplemented with 5% CO₂ in AnaeroPack with AnaeroPouch.
13. After 2-4 weeks, colonies of the transformed cells appear on the MA2 solid gellan gum plate.

Recipes

1. 20% slurry cornstarch stock solution (Fujiwara and Ohnuma, 2018)
 - a. Add 10 g of cornstarch and 40 ml of dH₂O to the 50 ml conical tube
 - b. Mix the tube well using a vortex mixer
 - c. Centrifuge the tube at 1,200 $\times g$ for 5 min at 4 °C
 - d. Discard the supernatant by decantation
 - e. Resuspend the pellet in 40 ml of 100% of ethanol using a vortex mixer
 - f. Centrifuge the tube at 1,200 $\times g$ for 5 min at 4 °C
 - g. Discard the supernatant by decantation
 - h. Fill up to 50 ml with 75% of ethanol
 - i. Store the tube at 4 °C, wrapped using parafilm until use
2. MA2 medium
 - a. Mix 100 ml of MA2 solution I, 10 ml of MA2 solution II, 1 ml of MA2 solution III and 885 ml of dH₂O in a glass bottle and sterilize by autoclaving

b. Add 4 ml of MA2 solution IV to the mixture in a laminar flow cabinet

MA2 solution I

Components	Amount	Conc.	Final conc. in MA2
(NH ₄) ₂ SO ₄	52.9 g	400 mM	40 mM
MgSO ₄ ·7H ₂ O	9.9 g	40 mM	4 mM
H ₂ SO ₄	3 ml	0.3% (v/v)	0.03% (v/v)
A6 minor salts	40 ml		
H ₂ O	Up to 1,000 ml		

A6 minor salts

Components	Amount	Conc.	Final conc. in MA2
H ₃ BO ₃	2.85 g	46 mM	184 µM
MnCl ₂ ·4H ₂ O	1.8 g	9 mM	36.4 µM
ZnCl ₂	0.105 g	0.77 mM	3.08 µM
Na ₂ MoO ₄ ·2H ₂ O	0.39 g	1.6 mM	6.44 µM
CoCl ₂ ·6H ₂ O	0.04 g	0.17 mM	6.72 µM
CuCl ₂ ·2H ₂ O	0.043 g	0.25 mM	1.01 µM
H ₂ O	Up to 1,000 ml		

MA2 solution II

Components	Amount	Conc.	Final conc. in MA2
KH ₂ PO ₄	10.9 g	800 mM	8 mM
H ₂ O	Up to 100 ml		

MA2 solution III

Components	Amount	Conc.	Final conc. in MA2
CaCl ₂ ·2H ₂ O	14.7 g	1 M	1 mM
H ₂ O	Up to 100 ml		

MA2 solution IV

Components	Amount	Conc.	Final conc. in MA2
FeCl ₃ ·6H ₂ O	0.68 g	25 mM	0.1 mM
Na ₂ EDTA	0.74 g	20 mM	0.075 mM
H ₂ SO ₄	3 drops		
H ₂ O	Up to 100 ml		

Note: This MA2 solution IV should be sterilized by filtration and stored at 4 °C in the dark.

3. MA2 solid gellan gum plate (for making 9-10 plates)
 - a. Mix 30 ml of MA2 solution I, 3 ml of MA2 solution II, 300 µl of MA2 solution III, and 70 ml of dH₂O in a 500 ml flask. Mix 200 ml of dH₂O and 1.5 g of gellan gum in another 500 ml flask. Sterilize these flasks by autoclaving, and subsequent plate preparation should be performed in a laminar flow cabinet
 - b. After autoclaving, mix both solutions and add 1.2 ml of MA2 solution IV. In case of 5-FOA selection to isolate the *URA* deficient mutants (for example applying *URA* marker recycling system, Takemura *et al.*, 2018; Takemura *et al.*, 2019), add directly 150 mg of uracil (final concentration: 0.5 mg/ml) and 240 mg of 5-FOA (final concentration: 0.8 mg/ml) in powder to the flask
 - c. Pour the mixed solution into the sterilized plastic plates
 - d. Dry the plates at room temperature for 15 min
 - e. Store at 4 °C in the dark

Acknowledgments

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Competing interests

No competing interests declared.

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I Plate-based Assay for Studying How Fungal Volatile Compounds (VCs) Affect Plant Growth and Development and the Identification of VCs via SPME-GC-MS

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[Abstract] Biogenic volatile compounds (VCs) mediate various types of crucial intra- and inter-species interactions in plants, animals, and microorganisms owing to their ability to travel through air, liquid, and porous soils. To study how VCs produced by *Verticillium dahliae*, a soilborne fungal pathogen, affect plant growth and development, we slightly modified a method previously used to study the effect of bacterial VCs on plant growth. The method involves culturing microbial cells and plants in I plate to allow only VC-mediated interaction. The modified protocol is simple to set up and produces reproducible results, facilitating studies on this poorly explored form of plant-fungal interactions. We also optimized conditions for extracting and identifying fungal VCs using solid phase microextraction (SPME) coupled to gas chromatography-mass spectrometry (GC-MS).

Keywords: *Arabidopsis thaliana*, GC-MS, I plate, Plant-fungal interaction, SPME, *Verticillium dahliae*, Volatile compounds

[Background] Volatile compounds (VCs) have been shown or suggested to play varied and crucial roles in mediating organismal interactions within and across kingdoms. Plants rely on VCs to attract pollinators, seed dispersers, and parasitoids (Baldwin, 2010; Herrmann, 2010). Animals have evolved sophisticated olfactory systems to detect and respond to foods, threats, and mates through volatile cues (Buck, 2004). Similarly, microbial VCs seem to perform diverse functions such as suppressing competitors, regulating their population density, and controlling morphological transitions (Bailly and Weisskopf, 2012; Bennett *et al.*, 2012; Bitas *et al.*, 2013). Roles of microbial VCs in plant growth, development, and stress response have been investigated using several experimental setups that physically separate microbial cells from plants so that only VC-mediated interaction can occur (Ryu *et al.*, 2003; Kai and Piechulla, 2009; Xie *et al.*, 2009; Hung *et al.*, 2013; Vaishnav *et al.*, 2015). Among them, a method employing bipartite Petri plate, also called I plate, has been most frequently used. We adopted I plate to study the effect of VCs produced by soilborne fungal pathogens on plant growth, development, and responses to biotic and abiotic stresses (Bitas *et al.*, 2015; Li and Kang, 2018; Li *et al.*, 2018b). In addition, we optimized a scheme for VC extraction and analysis to help identify fungal VCs that are responsible for modulating plant growth and development (Li *et al.*, 2018b).

Here, we provide a detailed protocol for setting up an I plate assay used for evaluating the effect of

VCs produced by *Verticillium dahliae*, a devastating soilborne fungal pathogen that infects hundreds of plant species, on *Arabidopsis thaliana*. This protocol enables rapid and straightforward determination of if and how fungal VCs affect plants. We also describe a protocol for capturing VCs through solid phase microextraction (SPME) and analyzing extracted VCs via gas chromatography-mass spectrometry (GC-MS). In combination, these protocols will help explore how VCs produced by diverse fungi affect plants and can also be applied to study VC-mediated interactions between microbes.

Materials and Reagents

1. Surgical blades #10 and #11
2. Parafilm (Bermis, catalog number: PM-99)
3. Paper towel
4. 10 µl and 1,000 µl micropipette tips
5. 100 x 15 mm I plate (VWR, catalog number: 25384-310)
6. 100 x 100 mm square plate (VWR, catalog number: 10799-140)
7. 100 x 15 mm (VWR, catalog number: 25384-302) and 60 x 15 mm (VWR, catalog number: 25384-092) Petri plates
8. 1.7 ml microcentrifuge tube (VWR, catalog number: 87003-294)
9. 25 ml serological pipette (VWR, catalog number: 89130-900)
10. Filter unit with 0.2 µM cellulose membrane (Nalgene, catalog number: 121-0020)
11. 1.5 ml sample vial (Shimadzu, catalog number: 221-34274-91), white cap with septum
12. *V. dahliae* strains PD322 and PD413 (conidial suspension in 20% glycerol and stored at -80 °C)
13. *A. thaliana* ecotype Col-0 seeds (Lehle Seed Co.)
14. Sterile MilliQ water
15. Murashige and Skoog (MS) basal medium (Sigma-Aldrich, catalog number: M0404-10L)
16. Sucrose (Alfa Aesar, catalog number: A15583)
17. Granulated agar (Difco, catalog number: 214530)
18. Potato dextrose agar (PDA) (Difco, catalog number: 213400)
19. 200 proof ethanol (KOPTEC, catalog number: 64-17-5)
20. 6% sodium hypochlorite (CLOROX)
21. n-Hexane (EMSURE, catalog number: 1043744000)
22. C₇-C₃₀ saturated alkanes (Sigma-Aldrich, catalog number: 49451-U)
23. 99.99% pure helium gas
24. 0.5x PDA medium (see Recipes)
25. MS agar medium (see Recipes)

Equipment

1. Pyrex glass bottle

2. 10 μ l and 1,000 μ l micropipettes
3. Scalpel
4. Forceps
5. Cork borer (5 mm in diameter)
6. SPME fiber holder (Supelco, catalog number: 57330-U)
7. 15 ml clear glass vial (Supelco, catalog number: 27159), screw cap with PTFE/silicone septum
8. SPME fiber assembly with 50/30 μ m DVB/CAR/PDMS fiber coating (Supelco, catalog number: 57328-U)
9. Electric pipette controller (Drummond Scientific Co., catalog number: 4-000-110-TC)
10. Vortex (VWR, model: Genie 2)
11. Table-top centrifuge (Eppendorf, model: 5417C)
12. Table-top shaker (VWR, catalog number: 57018-754)
13. Analytical balance (Mettler Toledo, model: AE-100)
14. Dissecting microscope (Zeiss, model: Stemi 2000-C)
15. Incubator (Sheldon Manufacturing, model: 1510E)
16. Plant growth chamber (Conviron, model: CMP5090)
17. Flexible-arm electrode holder (Mettler Toledo, catalog number: 30266628)
18. GC-MS system (Shimadzu, model: GCMS-QP2010 ultra) equipped with AOC-20i auto injector (Shimadzu, catalog number: 221-72315-48)
19. Rtx-Wax capillary (60 m, 0.25 mm ID and 0.25 μ m d_f) column (Restek, catalog number: 12426)
20. 4 °C refrigerator
21. Autoclave

Software

1. ImageJ (version 1.52a)
2. GC-MS Solution (Shimadzu, version 2.72), a package supporting GC-MS real-time and post-run analyses
3. National Institute of Standards and Technology (NIST) Mass spectral library (Shimadzu, version 11)

Procedure

A. I plate assay (Figure 1)

1. Use a sterile 10 μ l pipette tip to streak *V. dahliae* stock on 0.5x PDA (Recipe 1) plate in a zigzag pattern and incubate at 22 °C for 10 days.
2. Surface sterilize *A. thaliana* seeds as follows:
 - a. Add 1 ml 95% ethanol to a 1.7 ml microcentrifuge tube containing seeds, vortex, and incubate for 1 min.

- b. After removing ethanol, wash once with sterile MilliQ water and discard the water.
 - c. Add 1 ml 6% sodium hypochlorite solution, vortex, and incubate for 15 min by shaking at 100 rpm.
 - d. Wash twice with sterile MilliQ water after removing the sodium hypochlorite solution.
 - e. Incubate seeds in 1 ml sterile MilliQ water at 4 °C in darkness for 3 days.
3. Prepare square plates with MS agar (Recipe 2) and slice the medium into 10 x 10 mm pieces using a sterilized scalpel with blade #10.
 4. Hold one seed at the tip of a 10 µl micropipette using suction and then release the seed onto each agar piece. Seal the plate with two layers of Parafilm and place in a plant growth chamber set at 22 °C, 12 h light (4,500 lux, 60 µmol photons m⁻² s⁻¹), and 60% relative humidity for 7 days.
 5. Prepare I plate by adding 8 ml MS agar to one compartment and 8 ml 0.5x PDA to the other compartment.
 6. Transfer five *A. thaliana* seedlings (similar in size and growth stage) along with attached agar piece to the MS side of I plate using a sterilized scalpel with blade #10.
 7. Use a heat-sterilized cork borer to generate culture plugs along the actively growing margin of *V. dahliae* culture and place one plug (upside down) to the far end of the PDA side of I plate using a sterilized scalpel with blade #11.
 8. Seal the inoculated I plate with two layers of Parafilm and place in a plant growth chamber for a designated amount of time for each experiment (see Li et al., 2018b for specific examples).

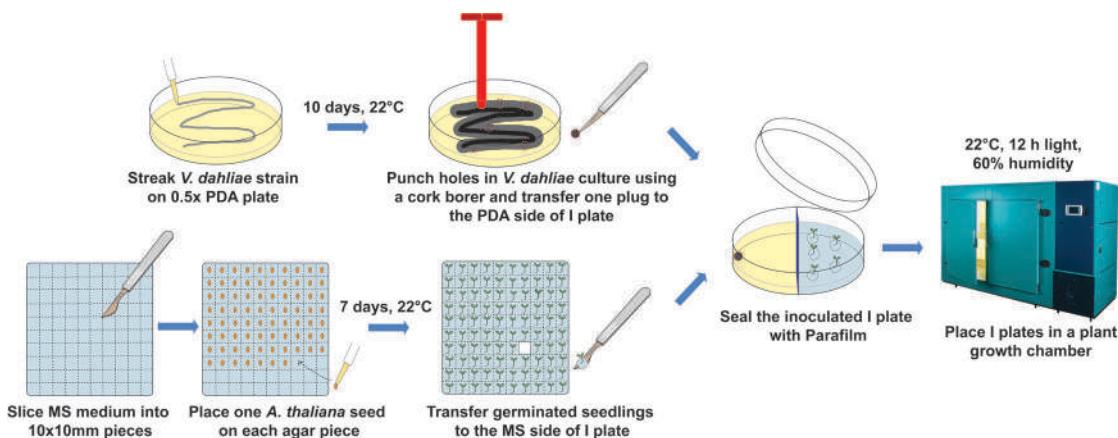


Figure 1. Workflow of the I plate-based assay

B. Extraction of VCs produced by *V. dahliae* (Figure 2)

1. Inoculate a plug of *V. dahliae* culture on 8 ml PDA slant in a 15 ml clear glass vial. Seal the vial with Parafilm.
2. Incubate at 22 °C until the culture fully covers the surface of PDA slant, which typically takes 8 days.
3. Replace Parafilm with a screw cap containing PTFE/silicone septum and incubate for one day.
4. Condition the SPME fiber before VC extraction by placing the SPME needle into the GC injection

port set at 230 °C for 1 h.

- Insert the conditioned SPME fiber in the injection port and starting the GC temperature program shown in Table 1. Desorb the fiber for 5 min in the injection port. Retract the fiber and remove the needle after 5 min and check resulting chromatograms when the program is completed. The intensity of background peaks (= blank sample) should be very low. Otherwise, repeat the desorption of the fiber.
- Extract VCs for 1 h by leaving the SPME fiber in the headspace of sampling vial. Use a flexible-arm electrode holder to lock the position of the SPME fiber holder so that the insertion depth of fiber is uniform for all extractions.

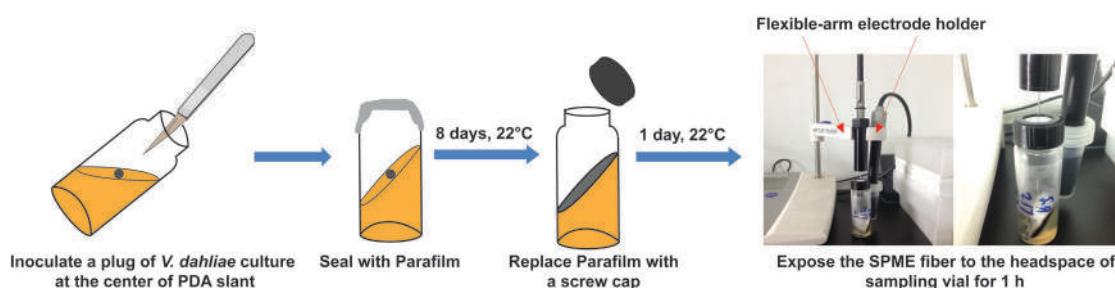


Figure 2. Workflow of volatile compound extraction

Table 1. Conditions for gas chromatography-mass spectrometry analysis

Column	Type	Rtx-Wax capillary column
	Length (m)	60
	ID (mm)	0.25
	D _f (μm)	0.25
Carrier Gas	Gas type	Helium (99.99% pure)
	Total flow	10 ml min ⁻¹
	Flow rate	1 ml min ⁻¹
	Purge flow	4 ml min ⁻¹
	Pressure	109.2 kPa
Inlet	Temperature	230 °C
	Injection mode	Splitless
Oven Temp. Program	Isothermal heating	5 min at 35 °C
	Temperature gradient	5 °C min ⁻¹ to 230 °C
	Final heating	15 min at 230 °C
Mass Spectra	Mass scan method	Total-ion-count (TIC)
	Mass scan range	35 to 500 m/z
	Run time	1.5 to 59.0 min
	Event time	0.3 s

C. GC-MS analysis of extracted VCs (Figure 3)

- Analyze the extracted VCs using a GC-MS system (manual injection mode, Figure 3A) by following the conditions described in Table 1. Insert the SPME needle into the GC injection port immediately after retracting it from each sampling vial. Desorb VCs bound to the SPME fiber for 5 min. An Rtx-Wax capillary column is used as it exhibits better retention and separation of polar VCs.
- Place the fiber in the GC injection port for 20 min for desorption before extracting another sample of VCs.
- Analyze C₇-C₃₀ saturated alkane standards using the automatic sample injection mode (Figure 3B). Connect the AOC-20i auto injector to the GC injection port. Place a 1.5 ml sample vial containing 0.5 ml solution of standards (1:50 dilution with n-hexane) on the rack of the auto injector. Inject 1 μ l solution into the GC-MS system and analyze using the same column and conditions (Table 1).

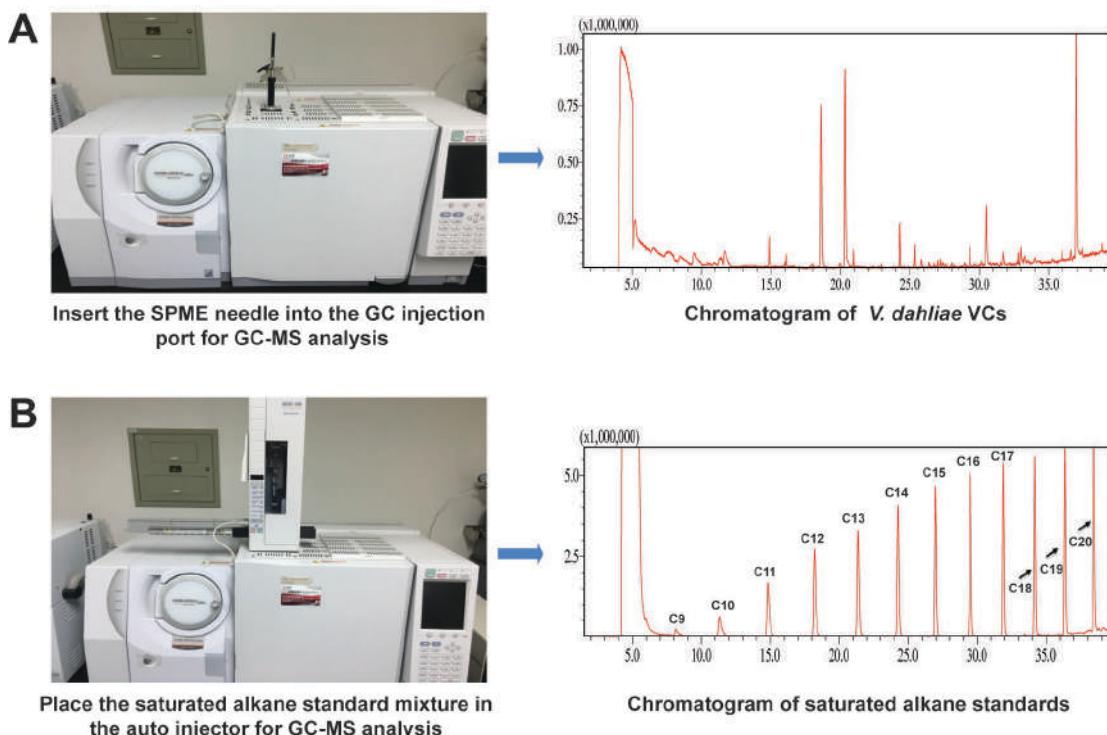


Figure 3. Gas chromatography-mass spectrometry analysis. A. Extracted volatile compounds (VCs) from *V. dahliae* were analyzed via the manual injection mode. B. Alkane standards were analyzed using the automatic sample injection mode.

Data analysis

- Assessment of root growth and development after individual VC treatments
 - After co-cultivation, use forceps to gently pull *A. thaliana* seedlings from the medium without damaging their roots.

2. Remove excess moisture on the roots using a paper towel.
3. Detaching roots from the shoot using forceps, weigh the roots immediately using an analytical balance.
4. Mount the roots on a flat surface (e.g., 100 x 15 mm Petri plate) and add 3 ml water on them.
5. Gently spread the roots using forceps so that the primary and lateral roots do not overlap.
6. Use the bottom of 60 x 15 mm Petri plate to flatten the roots (Figure 4A) for subsequent measurements:
 - a. Count the number of lateral roots, including all branches, under a dissecting microscope.
 - b. Take pictures of the roots and import them to ImageJ. Follow the instruction in ImageJ to measure the primary root length (Figure 4B).
7. Calculate the lateral root density of each sample by dividing the number of lateral roots by the primary root length.

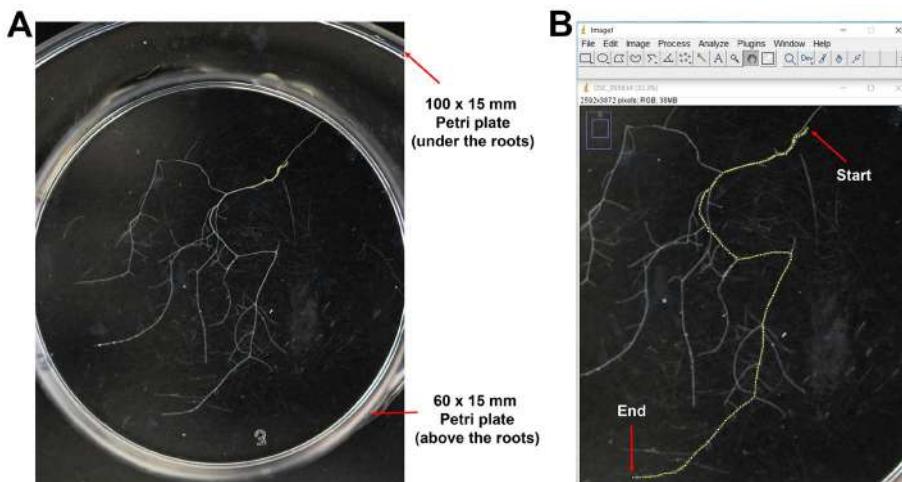


Figure 4. Analysis of *A. thaliana* roots after treating with volatile compounds produced by *V. dahliae*. A. Flattened *A. thaliana* roots by pressing with a 60 x 15 mm Petri plate. B. Snapshot of ImageJ data used to measure the length of the primary root (indicated by the yellow line) after VC treatment. The start and end of the primary root were indicated using red arrows.

B. Identification of individual compounds

1. Peak finding, peak integration, and retention time correction were performed using the post-run analysis software in the GC-MS Solution package.
2. The putative identity of each compound (peak) was determined using the following methods.
 - a. Compare resulting mass spectral profiles with reference data archived in the NIST mass spectral library (version 11). The top hit(s) with match factors $\geq 90\%$ were put on a “positive list” of tentatively identified compounds.
 - b. Calculate the retention index (RI) of each compound using the following equation (for temperature programmed chromatography) (Kováts, 1958).

$$RI_x = 100 \left[\frac{(Tx - Tn)}{(TN - Tn)} + n \right]$$

where,

x = Unknown compound in the sample

n = The number of carbons in the alkane preceding the unknown compound

N = The number of carbons in the alkane following the unknown compound

T_x = The retention time of the unknown compound

T_n = The retention time of the preceding alkane

T_N = The retention time of the following alkane

The experimentally obtained RI of each peak was compared to those in the NIST Chemistry WebBook (<https://webbook.nist.gov>, using polar columns). For positive confirmation of identity, a maximum relative deviation of $\pm 2\%$ from published values was used (Stoppacher *et al.*, 2010).

Notes

1. The I plate assay may not be suitable for studying the effect of VCs produced by fungi that grow rapidly, as fungal mycelia may grow over the central divider of I plate and contaminate the MS medium. We removed agar strips from both sides of the divider of I plate to prevent this kind of contamination when we studied fungi that grow faster than *V. dahliae* (Bitas *et al.*, 2015). However, for fungi like *Trichoderma*, this measure was not sufficient (Li *et al.*, 2018a).
2. Due to the limited space of I plate, only small plants (e.g., *A. thaliana* and *Nicotiana benthamiana*) in their early growth stage are suitable for this assay.
3. Under the growth conditions used, growth promoting effect on *A. thaliana* becomes noticeable as early as after 7 days of co-cultivation with *V. dahliae* (Li *et al.*, 2018b).
4. Because *A. thaliana* starts to initiate inflorescence development after 14 days of co-cultivation, we recommend that the duration of co-cultivation should not exceed 14 days.
5. Here, we only described how to analyze the root growth and development after VC exposure. Other traits of VC-exposed plants, such as shoot weight, chlorophyll content, physiological and molecular changes, can also be analyzed (e.g., Zhang *et al.*, 2007 and 2008; Li *et al.*, 2018b).
6. Several SPME fibers and GC columns may need to be evaluated to optimize the scheme for VC extraction and analysis. In our research, the DVB/CAR/PDMS 50/30 μm fiber coating extracted the largest number of VCs, and the Rtx-Wax capillary column enabled better separation of extracted VCs than the DB-5 column.
7. Analysis of VCs extracted from uninoculated PDA slant is necessary to exclude VCs derived from the medium and the environment.

Recipes

1. 0.5x PDA medium (1 L)
19.5 g PDA
Pour the autoclaved medium after cooling it down to 50 °C
2. MS agar medium (1 L)
4.5 g MS and 7.5 g agar
Add 5 ml 50% (w/v) sucrose (filter sterilized) after cooling down the autoclaved medium to 50 °C
Mix well before pouring

Acknowledgments

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Competing interests

The authors declare no conflicts of interest or competing interests.

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Peptide Feeding and Mechanical Wounding for Tomato Seedlings

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[Abstract] Plants need to respond appropriately to wounding and herbivorous insects. Peptide signals have been implicated in local and systemic induction of appropriate plant defense responses. To study these peptide signals and their perception in host plants, it is important to have reproducible bioassays. Several assays, such as treatment of peptide solution via pressure infiltration, have been developed. Here, we provide detailed protocols for peptide feeding and mechanical wounding for tomato seedlings. To directly introduce peptides into tomato seedlings, peptide solution is fed through the excised stem via the transpiration stream. To mimic the wounding caused by insect feeding, leaflets of tomato seedlings are mechanically damaged with a hemostat; and wounded and systemic unwounded leaves are harvested and analyzed separately. Samples from both assays may be further assessed by examining the transcript level of marker genes by quantitative real-time PCR (qRT-PCR).

Keywords: Tomato, Systemin, Peptide signaling, Wounding, Wound response, Plant-insect interaction

[Background] Peptide signaling plays crucial roles in regulating plant growth, development and interaction with the surrounding environment (Tavormina *et al.*, 2015). Perception of the peptide systemin, for example, is critical for resistance against chewing insect larvae (Pearce *et al.*, 1993; Wang *et al.*, 2018). Attack by such larvae and related wounding lead to various immune responses, one of which is the induction of defense-related marker genes. In tomato, the genes encoding proteinase inhibitor 1 (PIN1) and proteinase inhibitor 2 (PIN2) were shown to be highly activated after insect feeding or wounding, and have been extensively used as hallmarks for defense triggered by insects or wounding (Pearce *et al.*, 1991; Ryan and Pearce, 2003). Here we provide a protocol focusing on details of peptide feeding and wounding assays, which can be followed by quantification of *PIN1* transcript via qRT-PCR. The protocol has been optimized to minimize variations inherent in these assays and was previously used to assess the function of systemin perception (Wang *et al.*, 2018).

Materials and Reagents

1. 12 mm x 75 mm glass test tubes (VWR, catalog number: 212-0013) (plastic tubes also work)
2. Latex gloves or nitrile gloves
3. 5 ml pipette tips (Kinesis, catalog number: U696256)
4. Aluminum foil
5. Single edged steel razor blades (Carl Roth, catalog number: CK07.1)
6. 9.5 cm round plant pots with a volume of 0.32 L (Gartnereinkauf, catalog number: 410103) (pots

with similar size or larger also work)

7. 6.5 cm round plant pots with a volume of 0.1 L (Gartnereinkauf, catalog number: 410066)
8. Plant seedling propagation trays with clear lids (60 cm x 40 cm x 6 cm) (meyer-shop, catalog number: 749114) (other similar trays also work)
9. 5 ml pipette (Kinesis, catalog number: 2400631)
10. Tomato seeds
11. Potting soil (Pikiererde and Topferde ordered from Einheitserde, Germany; mix 1:1) (other potting soil with medium to high nutrient and fine texture also works)
12. Systemin peptide (peptide sequence: AVQS KPPC KRDP PKMQ TD) (GenScript, catalog number: 88910480001)
13. Sterile water
14. 70% ethanol
15. Liquid nitrogen

Equipment

1. 5.5-inch hemostat with serrated jaws (Merck, catalog number: Z168858-1EA)
2. Long tweezers (Carl Roth, catalog number: PT95.1)
3. Scissors
4. Plastic racks for test tubes
5. -80 °C freezer
6. -20 °C freezer

Procedure

A. Peptide feeding assay for tomato seedlings

1. Sow tomato seeds on a single 9.5 cm diameter pot filled with potting soil. Cover seeds with ~5 mm soil. Place the pot in a tray filled with ~1 cm water. Cover the tray with a transparent plastic lid. Place the tray in a greenhouse with the following conditions: 14 h light/10 h dark; 25 °C/19 °C day/night; 30%-40% relative humidity; 12.38 klx. Remove the lid 1 day after seed germination.
Note: Covering seeds with a thin layer of soil will help remove seed coats from cotyledons during germination. Twelve tomato seedlings are required for each treatment. To guarantee enough seedlings of the desired size, it is recommended to sow 50% more seeds.

2. Around 10 days after sowing, separate individual tomato seedlings of similar size into 6.5 cm diameter pots when the first true leaves are emerging (Figure 1). Leave as much hypocotyl above the soil as possible during transplanting. Place the pots in a tray, and water the pots thoroughly from the bottom by adding water on the tray. Cover the tray with a clear lid.

Note: A long hypocotyl will reduce the chance that the tomato seedlings lose contact with peptide solution/water during the feeding assay.



Figure 1. A freshly transplanted tomato seedling

3. Remove the lid after two days. Water plants daily from the bottom of the pots by adding water on the tray. Grow tomato seedlings until the third true leaves are emerging (~18 days after sowing).

Note: Seedlings of different tomato cultivars have different growth rate. Refer to the size of the seedlings rather than growing time.

4. The morning before the feeding assay, water the tomato seedlings thoroughly from the bottom. Do not water them after this time point.
5. Dissolve systemin peptide powder in sterile water to make a 10 mM stock. Dilute peptide stock solution to 100 nM with sterile water immediately before the feeding assay. Prepare 4 ml peptide solution for each seedling and 50 ml extra as backup. Store the remaining peptide stock at -20 °C.
6. Add 4 ml peptide solution (or sterile water as the control) into glass test tubes.
7. Excise tomato seedlings of similar size at their bases (~3 mm above the soil) with a razor blade and put the seedlings immediately into glass test tubes containing peptide solution (1 seedling per tube) (Figure 2). Wear gloves during treatment and always change gloves when switching solutions.

Note: When dealing with a large number of samples, estimate the time needed in advance for treating and harvesting every group of samples. Leave 10 min for buffering between two groups. Avoid cross-contamination and wounding of seedlings.

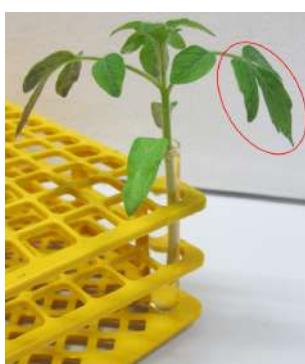


Figure 2. Tomato seedlings fed with peptide solution. The red circle indicates the leaf to be

harvested.

8. Feed seedlings with peptides (or water as the control) via the transpiration stream for 1 h in the greenhouse. Meanwhile, prepare new glass tubes fully filled with sterile water.
9. Transfer seedlings into new glass tubes. Avoid cross-contamination of peptide solutions by switching gloves between different sets of treatment.
10. Incubate excised seedlings in the greenhouse for 8 h. Check liquid level every 2 h. Refill the tubes with sterile water once they are half empty.
Note: Due to differences in solution uptake, some plants may wilt slightly at first. They will recover after further incubation.
11. Harvest 1 leaf from the first pair of true leaves with scissors. For each treatment, pool leaves from 3 plants as 1 biological sample and prepare 4 biological samples.
Note: The amount of peptide taken up through transpiration stream may differ greatly among different plants. It is thus important to use pooled samples as biological replicates to minimize variation.
12. Wrap leaf samples quickly with aluminum foil. Freeze the samples immediately in liquid nitrogen with tweezers. Store samples at -80 °C for further analysis.

B. Wounding assay for tomato seedlings

1. Prepare ~18 days old tomato seedlings in the same way as described in Steps A1-A3.
2. Water the plants thoroughly from the bottom of the pots in the evening before the wounding assay.
Note: It is important to have sufficient water in the soil before performing the wounding assay. The plants should not be watered during the assay.
3. Early the second morning, pinch the leaflets of one leaf from the first pair of true leaves with a hemostat twice at each side of the mid-rib, once at ~1/4 part of the leaflets and the other time at ~3/4 part. Supply enough pressure to wound the leaf surface but not too hard to penetrate the leaf. Clean the jaw of the hemostat with 70% ethanol between the different sets of treatment.
Note: Do not break the mid-rib during wounding; otherwise the leaflets will dry out by the end of the assay.
4. Three hours after the first wounding, pinch the same leaflets once again at ~1/2 part (Figure 3).



Figure 3. A tomato seedling with a leaf wounded by a hemostat. Red arrows indicate the leaflets of a wounded leaf, black arrows indicate the leaflets of a systemic leaf (unwounded leaf from the same plant).

5. Twelve hours after the first wounding, harvest the leaves with a pair of scissors. Pool 3 leaves (1 leaf per plant) from the same tomato line as a biological sample. Collect unwounded leaves from the treated plants as systemic leaves. Collect leaves from untreated plants as control leaves.

Note: PIN1 transcript levels in different tomato seedlings may vary greatly after induction. To reduce variation, it is important to pool several plants as a biological sample. Prepare 4 biological samples per treatment.

6. Wrap leaf samples quickly with aluminum foil and freeze the samples immediately in liquid nitrogen with tweezers. Store samples in a -80 °C freezer for further analysis.

Acknowledgments

This protocol is partially adapted from Pearce *et al.* (1993). This work was funded by the grant DFG-CRC1101-D05.

Competing interests

The authors declare no competing interests.

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Quantitative Plasmodesmata Permeability Assay for Pavement Cells of *Arabidopsis* Leaves

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[Abstract] Plasmodesmata (PD) are intercellular channels between walled plant cells that enable the transportation of materials between adjacent cells, which are important for plant growth and development. The permeability of PD must be tightly regulated. Assays to determine the permeability of PD are crucial for related studies on the regulation of PD development and permeability. Here we describe an assay for the determination of PD permeability via the observation and quantification of GFP diffusion and cell-to-cell transport of CMV MP-GFP in *Arabidopsis* leaves.

Keywords: Plasmodesmata, PD permeability, GFP, CMV MP-GFP, Particle bombardment

[Background] Plasmodesmata (PD) are plant-specific channels between cells, which are important for plant growth and development as well as the interaction between plants and the surrounding environment (Maule et al., 2011; Lee, 2015; Cheval and Faulkner, 2018). Water and small molecules can pass through PD freely. Other macromolecules, e.g., proteins, RNA, and some pathogens, can also move via PD. However, the mobility of those molecules between adjacent cells is tightly regulated, although the mechanism underlying this transport is not well understood (Sevilem et al., 2015). Currently, there are several approaches reported for measuring the permeability of PD in plant tissues. One of the methods is to visualize the diffusion of fluorescent dextrans or other fluorescent probes from the targeted cells into neighboring cells (Ding et al., 1996). This approach allows the researchers to select fluorescent dextrans of different sizes which will facilitate the study of PD permeability. In addition, this method also allows researchers to perform the coinjection of fluorescent dextrans with special drugs or proteins that allows the colleagues to assay the effect of those drugs or proteins on the permeability of PD. However, this method requires a rigorous experimental system and a highly experienced operator to perform the experiments. The second method is Drop-AND-See (DANS) which uses the 5(6)-carboxy fluorescein diacetate (CFDA) for the rapid assessment of PD permeability (Cui et al., 2015). DANS is a simple and rapid approach, but it is impossible to trace the spread of CFDA at the single cell resolution. The researchers in this field also used the green fluorescent protein (GFP) (Crawford and Zambryski, 2001; Liarzi and Epel, 2005) and *Cucumber mosaic virus* (CMV) Movement Protein (MP)-GFP (CMV MP-GFP) (Iglesias and Meins, 2000) as probes to assay the permeability of PD. The expression of GFP or CMV MP-GFP was achieved by delivering plasmids expressing them into plant cells via particle bombardment. This method enables the researchers to visualize the diffusion of GFP from targeted cells into adjacent cells or cell-to-cell movement of CMV MP-GFP. Here, we introduce the detailed method of GFP diffusion

and cell-to-cell movement of CMV MP-GFP, which is adapted from our recently published paper (Diao et al., 2018).

Materials and Reagents

1. 9 cm glass dish (NORMAX, catalog number: 5058546)
2. Pipette tips (USA Scientific, catalog numbers: 1112-1720, 1110-1200)
3. Eppendorf tubes (Fisher Scientific, Fisherbrand™, catalog number: 05-408-129)
4. Macrocarriers (Bio-Rad, catalog number: 1652335)
5. 1,100 psi rupture discs (Bio-Rad, catalog number: 1652329)
6. Stopping screens (Bio-Rad, catalog number: 1652336)
7. Coverslip (THOMAS SCIENTIFIC, catalog number: 6672A46)
8. *Arabidopsis thaliana* plants
9. Tungsten M10 or M17 microcarriers (Bio-Rad, catalog numbers: 1652266 or 1652267)
10. TIANprep Midi Plasmid Kit (TIANGEN, catalog number: DP103-03)
11. Spermidine (Sigma-Aldrich, catalog number: S2626)
12. Calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) (Sigma-Aldrich, catalog number: C7902)
13. Plasmid pdGN (Lee et al., 2005)
14. Plasmid CMVMP-pdGN (Diao et al., 2018)
15. Bio-Rad 1.0 μm Gold Microcarriers (Bio-Rad, catalog number: 165-2263)
16. Ethanol (AMRESCO, catalog number: E193)
17. 50% sterile glycerin (Sigma-Aldrich, catalog number: G5516)
18. KNO_3 (Sigma-Aldrich, catalog number: P6083)
19. NH_4NO_3 (AMRESCO, catalog number: 94629)
20. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Sigma-Aldrich, catalog number: M1880)
21. KH_2PO_4 (Sigma-Aldrich, catalog number: P5655)
22. $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (Macklin, catalog number: M813652)
23. $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (Macklin, catalog number: Z820817)
24. H_3BO_4 (Sigma-Aldrich, catalog number: B6768)
25. KI (Sigma-Aldrich, catalog number: V900056)
26. $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (Sigma-Aldrich, catalog number: M1651)
27. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (Macklin, catalog number: C805354)
28. $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (Aladdin, catalog number: C8396-01)
29. Glycine (Sigma-Aldrich, catalog number: V900144)
30. VB1 (Sigma-Aldrich, catalog number: V-014)
31. VB6 (Sigma-Aldrich, catalog number: V-018)
32. Nicotinic Acid (Sigma-Aldrich, catalog number: V900424)
33. Inositol (Sigma-Aldrich, catalog number: I5125)
34. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (Macklin, catalog number: I809845)

35. Na₂-EDTA·2H₂O (AMRESCO, catalog number: 0105)
36. Sucrose (Sigma-Aldrich, catalog number: V900116)
37. Agar (Sigma-Aldrich, catalog number: A1296)
38. MS medium (see Recipes)
39. Gold suspension solution (see Recipes)

Equipment

1. Ophthalmic scissors
2. Ophthalmic tweezers
3. Centrifuge (Eppendorf, model: 5417R)
4. PDS-1000/He™ Biostatic Particle Delivery System (Bio-Rad, model: 1652257)
5. Plant LED Incubator
6. Zeiss LSM 510 META or an equivalent confocal microscope
7. Vortex (Corning, model: Corning® LSE™ Vortex Mixer)
8. NanoDrop 2000 (Gene Company Limited)
9. -20 °C freezer

Software

1. ImageJ (<https://imagej.nih.gov/ij/> version 1.51)
2. IBM SPSS Statistics (version 25)

Procedure

1. Prepare Petri dish containing 20 ml of MS medium with 0.8% agar.
2. *Arabidopsis* plants grown under the 16 h/8 h light/dark cycle for 3-4 weeks (Figure 1A). Cut rosette leaves of *Arabidopsis* plants that had not yet bolted by ophthalmic scissors and place in a Petri dish with the abaxial side facing upward as shown in Figure 1B.
Note: Cells of young leaves work better than old leaves. Leaves from different genotypes should be placed on MS medium equally divided (Figure 1B).

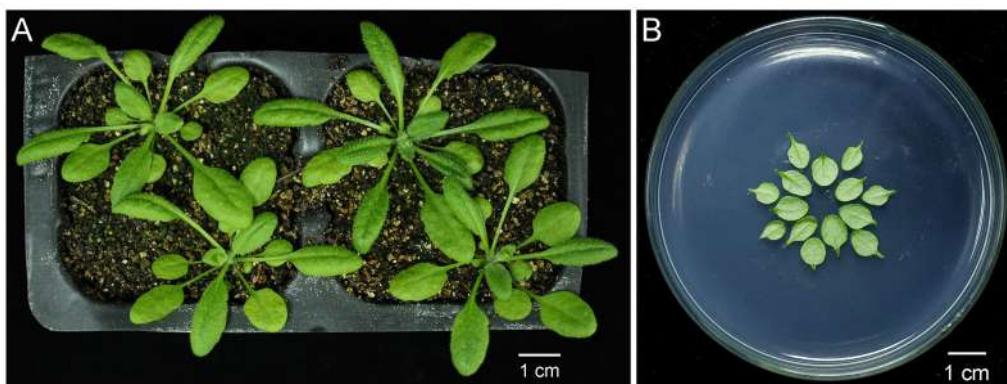


Figure 1. Preparation of the leaves of 4-week-old *Arabidopsis* plants for particle bombardment. A. *Arabidopsis* plants after growing under the 16 h/8 h light/dark cycle for 4 weeks. Scale bar = 1 cm. B. Rosette leaves that had not yet bolted were cut from *Arabidopsis* plants shown in (A) by ophthalmic scissors and placed concentrically with abaxial side facing upward on the surface of solid Murashige & Skoog medium. Scale bar = 1 cm.

3. Prepare pdGN plasmid from *E-coli* (Lee et al., 2005). Isolated the plasmid using A TIANprep Midi Plasmid Kit. The concentration of plasmid should be at least 1 µg/µl.

Note: The concentration of the plasmid is important for the next step. The pdGN-35S:HDEL-mCherry plasmid should also be prepared when we need to indicate the cells targeted by particle bombardment (Batoko et al., 2000).

4. Prepare bullets of biolistic bombardment. Vortex 10 µl gold suspension solution (Recipe 2) in a microcentrifuge tube with 2 µg plasmid DNA for 1 min. Add 20 µl fresh 0.1 M spermidine and vortex again for 1 min. Add 50 µl 2.5 M CaCl₂ and continue vortexing for another 3 min. Allow the mixture to settle for 5 min on ice and centrifuge the mixture for 10 s, at 2,348 x g. Discard the supernatant. Wash the pellet with 1 ml of 70% ethanol and then with 1 ml of 100% ethanol. Mix and centrifuge gently. Finally, resuspend the pellet in 15 µl 100% ethanol.

Notes:

- a. The maximum total volume of plasmid DNA should be ≤ 10 µl. When both pDGN plasmids and CMV MP-pDGN plasmids need to be expressed simultaneously, 2 µl of each and gold particles are mixed together in a single test tube.
 - b. The 1 M spermidine stock can be stored at -20 °C for at least 3 months.
5. Vortex the mixture gently and transfer it to a macrocarrier using a pipette with a 10-200 µl tip. Air dry until the ethanol has evaporated, assemble all of the parts necessary for performing a particle bombardment according to the manufacturer's instructions (Figure 2). Set the pressure to 1,100 psi and use 1,550 psi rupture discs. Place the MS plate with *Arabidopsis* leaves 9 cm below the macrocarrier (level 3) in the bombardment chamber (Figure 2). Set the vacuum level in the bombardment chamber to 28 psi and bombard.



Figure 2. Side view of PDS-1000/He™ Biostatic Particle Delivery System. Before performing particle bombardment, the Petri dish containing *Arabidopsis* leaves was moved into the PDS-1000/He™ Biostatic Particle Delivery System (left). Part 1 and part 2 of the system were zoomed in at right. a is 1,100 psi rupture disk. b is microparticles carrier disk, which carries 1.0 µm Gold Microcarriers. c is stopping screen.

6. Incubate the bombarded leaves for 24 h or 48 h at 23 °C in the dark.
7. Mount the bombarded leaves on a glass slide with a coverslip to image with a Zeiss LSM 510 META or an equivalent confocal microscope. The GFP signal is excited by a 488 nm argon laser and emission is captured in the range of 505–545 nm; the mCherry signal is excited by a 543 nm HeNe laser and the emission is captured in the range of 590–625 nm. Use a 20x/0.25 Fluar objective lens to scan the GFP cell clusters. Twenty percent laser intensity is used to image every fluorescent cell clearly.

Note: Acquire at least 60 images from each genotype for statistical analysis.

Data analysis

1. Open each image in ImageJ and find the cell expressing GFP (generally the cell with strongest fluorescence) which is defined as layer 0 (the edge of the cell is marked with red line in Figure 3). GFP protein diffuses from the layer 0 cell into other cells around it via PD. The cells that share a common cell wall with layer 0 cells were defined as layer 1 (the edge of cells is marked with orange lines in Figure 3). Cells that share a common cell wall with layer 1 cells, but not with layer 0 cells, were defined as layer 2 cells (the edge of cells is marked with blue lines marked in Figure 3). Cells that expressed GFP but showed no diffusion were not counted to avoid the situation in which damage was caused by the bombardment.

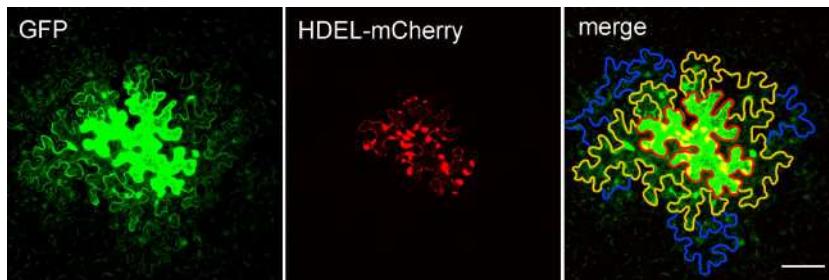


Figure 3. *Arabidopsis* leaf epidermal cells expressing *pdGN-35:GFP* and *pdGN-35S:HDEL-mCherry*. HDEL-mCherry is non-mobile, and was used to indicate the bombarded cell. The edge of the layer 0 cell is marked by a red line. The edges of the layer 1 cells and layer 2 cells are marked by orange and blue lines, respectively. Scale bar = 50 μ m.

2. Count the number of cell layers in the cell clusters. As the bombardment will cause damage for cells or induce the non-uniform expression of GFP that prevent the subsequent analysis, we normally capture at least 60 images from more than 6 plants per set but only pick data from three plants with healthy cells and uniform expression of GFP for the statistical analysis. Repeat the experiment at least three times.
3. The IBM SPSS Statistics (version 25) software was used to perform the statistical analysis. First, the normality of the datasets was assessed by Shapiro-Wilk tests (Shapiro and Wilk, 1965) the data were not normally distributed by Shapiro-Wilk tests, the Mann-Whitney *U*-test (Fay and Proschan, 2010) was applied for the subsequent statistical analyses.

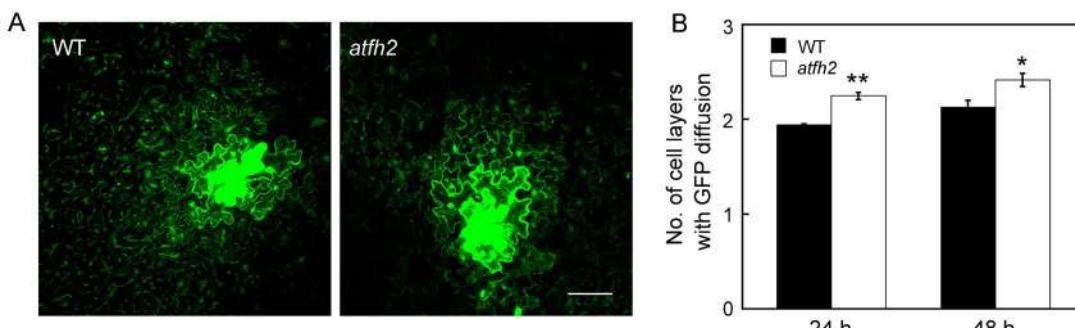


Figure 4. AtFH2 redundantly regulate PD permeability. A. Images of GFP diffusion in leaf epidermal pavement cells of WT and *atfh2* plants. Scale bar = 50 μ m. B. Quantification of the number of GFP diffusion layers in leaves of WT and *atfh2* plants. Values represent mean \pm SE. * P < 0.05, and ** P < 0.01 by Mann-Whitney *U*-test.

Recipes

1. MS medium (1 L)
76 g/L KNO₃
66 g/L NH₄NO₃

14.8 g/L MgSO₄·7H₂O

6.8 g/L KH₂PO₄

13.28 g/L CaCl₂

1.69 g/L MnSO₄·H₂O

8.6 g/L ZnSO₄·7H₂O

6.3 g/L H₃BO₄

0.83 g/L KI

0.25 g/L Na₂MoO₄·2H₂O

0.025 g/L CuSO₄·5H₂O

0.025 g/L CoCl₂·6H₂O

0.2 g/L Glycine

0.1 g/L VB1

0.05 g/L VB6

0.05 g/L Nicotinic Acid

10 g/L Inositol

2.78 g/L FeSO₄·7H₂O

3.73 g/L Na₂·EDTA·2H₂O

10 g/L Sucrose

6 g/L Agar

2. Gold suspension solution

- a. Place 30 mg of gold microcarriers into a 1 ml microcentrifuge tube
- b. Add 1 ml of 70% ethanol and vortex for 3 min
- c. Allow the mixture to settle for 15 min and centrifuge the mixture for 5 s
- d. Remove the supernatant and add 1 ml sterile water and repeat the previous step two times
- e. Remove the supernatant and add 500 µl 50% sterile glycerin
- f. Vortex the mixture gently and store at -20 °C

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Competing interests

The authors declared that they have no conflicts of interest to this work.

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Visualization of Plant Cell Wall Epitopes Using Immunogold Labeling for Electron Microscopy

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[Abstract] Plant cell walls consist of different polysaccharides and structural proteins, which form a rigid layer located outside of the plasma membrane. The wall is also a very dynamic cell composite, which is characterized by complex polysaccharide interactions and various modifications during cell development. The visualization of cell wall components *in situ* is very challenging due to the small size of cell wall composites (nanometer scale), large diversity of the wall polysaccharides and their complex interactions. This protocol describes immunogold labeling of different cell wall epitopes for high-resolution transmission electron microscopy (TEM). It provides a detailed procedure for collection and preparation of plant material, ultra-thin sectioning, specimen labeling and contrasting. An immunolabeling procedure workflow was optimized to obtain high efficiency of carbohydrates labeling for high-resolution TEM. This method was applied to study plant cell wall characteristics in various plant tissues but could also be applied for other cell components in plant and animal tissues.

Keywords: Cell walls, Cell wall epitopes, Polysaccharides, Immunogold labeling, Transmission electron microscopy

[Background] Plant biomass is mainly composed of cell walls, which are widely used as an energy source in our daily life (Loqué *et al.*, 2015). At the microscopic scale, cell walls consist of cellulose microfibrils embedded in complex matrix polysaccharides (hemicelluloses, pectins) and structural proteins. Cellulose microfibrils (CMFs) are the largest wall polymers with a radius of 3-5 nm and many micrometers long (Cosgrove, 2005). The orientation of CMFs determines the direction of growth and cell anisotropy (Baskin, 2005), but the CMFs also interact with other wall components all together modifying the wall properties (reviewed in Majda, 2018; Majda and Robert, 2018). The study of cell wall composition has a long history, going back to when different chemicals were applied to bind to the wall composites; however, many of them had a wide range of targets (Wallace and Anderson, 2012; Voiniciuc *et al.*, 2018) and could be observed only via light microscope resolution. In contrast, the immunogold labeling is characterized by a high specificity of antibodies and high-resolution imaging, which can precisely localize the wall epitopes across the wall matrix (e.g., Majda *et al.*, 2017). Despite electron microscopy being a relatively old method, it is not broadly used for plant cell walls. The reason could be that it is time-consuming, requires long training and extensive preparation time. In this protocol, I will walk you through all the steps concerning sample preparation, specificity for all the reagents and

troubleshooting.

Materials and Reagents

1. Adhesion slides, Polysine, 25 x 75 x 1.0 mm (VWR, catalog number: 631-0107)
2. Aluminum foil
3. Centrifuge tubes 15 ml (PluriSelect, catalog number: 05-00002-01)
4. Centrifuge tubes 50 ml (sterile) (PluriSelect, catalog number: 05-00001-01)
5. Compressed air in the can (e.g., air duster PRF 4-44)
6. Disposable pH indicator paper (universal indicator paper) (Johnson, catalog number: 101.3C)
7. Disposable plastic Pasteur pipettes (BRAND, catalog number: 747750)
8. Double-edged razor blades (Personna, catalog number: 171930)
9. Embedding capsules (8 mm flat, polypropylene capsules) (TAAB, catalog number: C095)
10. Filter papers (circles, 150 mm Ø), (Whatman, catalog number: 1001150)
11. Glass vials with plastic snap-cap (ca. 41 x 24 mm) (Karl Hecht, catalog number: 2783/3), or clear glass vials with snap-cap (closed top, PE transparent, 18 mm, 500 ml 20 x 40 mm) (VWR, catalog number: 548-0555)
12. Grids for transmission electron microscopy, e.g., grid size 100 mesh x 250 µm pitch, nickel or copper (TAAB, maxtaform HF4, catalog number: GM021N; Sigma-Aldrich, catalog number: G1528; Agar Scientific, catalog number: G2500C)
13. Light-duty 3-Ply tissue wipers (VWR, catalog number: 82003824-CS)
14. Metal needle or dissection needle (VWR, catalog number: 10806-330)
15. Microcentrifuge tubes 1.5 ml (Sigma-Aldrich, catalog number: Z606340)
16. Microscope slides, 25 x 75 x 1.0 mm (VWR, catalog number: 48300-025 or Thermo Scientific, catalog number: 10144633CF)
17. Paper tags
18. Parafilm (Sigma-Aldrich, catalog number: P7543)
19. Pencil
20. Pipette tips
21. Plastic Petri dishes (Fisherbrand, catalog number: S33580A)
22. Protective gloves (Honeywell, catalog number: Dermatril 740)
23. Round silicone rubber (TAAB, catalog number: G082)
24. Single edge razor blades with aluminum spine (VWR, catalog number: 233-0156)
25. Slide labels (Agar Scientific) or one side adhesive paper
26. Square Petri dish (120 mm) (Corning, catalog number: BP124-05)
27. Transparent tape (e.g., scotch)
28. Waste containers
29. Agar, plant agar (Duchefa Biochemie, catalog number: 9002-18-0)
30. Bovine Serum Albumin (BSA), lyophilized powder ≥ 96% (agarose gel electrophoresis) (Sigma-

- Aldrich, catalog number: 9048-46-8)
31. Disodium phosphate (Na_2HPO_4) (store in ambient temperature: room temperature, approx. 21 °C) (Sigma-Aldrich, catalog number: 7558-79-4)
 32. Distilled water
 33. Ethanol laboratory reagent, absolute, ≥ 99.5% (flammable, stored in designated place) (Sigma-Aldrich, catalog number: 64-17-5)
 34. Formaldehyde (FA) (10 ml ampule of 16% methanol-free FA) (health hazards, store closed in ambient temperature, or open in cold room/refrigerator 4 °C) (Thermo Scientific, catalog number: 28908) or paraformaldehyde (PFA) for histology (CH_2O)_n, (store in cold room/refrigerator 4 °C) (J.T. Baker, catalog number: S898-07)
 35. Formvar solution (1% formvar in dichloroethane, for microscopy) (Sigma-Aldrich, catalog number: 63148-64-1)
 36. Glutaraldehyde (GA) (grade I, 1 ml ampule of 25% GA in H_2O , specially purified for use as an electron microscopy fixative, linear formula: $\text{OHC}(\text{CH}_2)_3\text{CHO}$ (health hazards, store closed in ambient temperature, or open in cold room/refrigerator 4 °C) (Sigma-Aldrich, catalog number: 111-30-8)
 37. Hydrogen chloride (HCl) (Sigma-Aldrich, catalog number: 7647-01-0)
 38. LR white resin medium grade–catalyzed (health hazards, store in cold room/refrigerator 4 °C) (TAAB, catalog number: L012)
 39. Monopotassium phosphate (KH_2PO_4) (Sigma-Aldrich, catalog number: 7778-77-0)
 40. Monosodium phosphate (NaH_2PO_4) (store in ambient temperature) (Sigma-Aldrich, catalog number: 7558-80-7)
 41. Murashige and Skoog basal medium (MS) (Sigma-Aldrich, catalog number: M5519)
 42. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: 7447-40-7)
 43. Primary antibodies (PlantProbes: www.plantprobes.net or the University of Georgia: www.ccrc.uga.edu)
 44. Secondary antibodies, e.g., EM Goat anti-Rat IgG (H+L): 10 nm Gold (BBI Solutions, catalog number: 014990) or EM Goat anti-Mouse IgG (H+L) 10 nm Gold (BBI Solutions, catalog number: EM.GMHL10)
 45. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: 7647-14-5)
 46. Sodium hydroxide (NaOH) (Sigma-Aldrich, catalog number: 1310-73-2)
 47. Sucrose (Sigma-Aldrich, catalog number: 57-50-1)
 48. Toluidine blue for microscopy (Sigma-Aldrich, catalog number: 6586-04-5)
 49. TopVision low melting point agarose (LMP agarose) (store in ambient temperature) (Thermo Scientific, catalog number: R0801)
 50. Tween 20 (Sigma-Aldrich, catalog number: P1379)
 51. Uranyl acetate (UA) (health hazardous, store in ambient temperature) (VWR, catalog number: 541-09-3)
 52. Half strength MS basal medium (see Recipes)

53. Paraformaldehyde-glutaraldehyde (4% PFA and 0.05% GA) fixative solution (see Recipes)
54. Phosphate buffer (PB), 0.1 M solution (pH = 7.2) (see Recipes)
55. Low melting point (LMP) agarose 1% solution (see Recipes)
56. Different ethanol concentrations 10%-95% (see Recipes)
57. Different LRW resin concentrations 10%-75% (see Recipes)
58. Toluidine blue solution (see Recipes)
59. Blocking Reagent (BR) 1% (see Recipes)
60. Antibodies solutions (see Recipes)
61. Phosphate Buffered Saline (PBS), 0.1 M solution (pH = 7.2) (see Recipes)
62. Uranyl acetate solution (5%) (see Recipes)

Equipment

1. Analytical balance
2. Autoclave (YPO, model: D66161)
3. Centrifuge (Marshall Scientific, Eppendorf, model: 5417C)
4. Conical flask
5. Desiccator (Thermo Fisher, model: 5311-0250)
6. Diagonal cutting pliers e.g., stanley diagonal cutting pliers, 5 (Robosource, catalog number: 15-11)
7. Diamond knife, e.g., ultra 45° (Diatome)
8. Flat beaker or crystallizer (with a wide diameter)
9. Forceps
10. Freezer (-20 °C)
11. Fume hood
12. Glass baker (50 ml)
13. Glass knife strips (Agar Scientific, catalog number: AGG336)
14. Glass knifemakers for histology knives (LKB, catalog number: LKB 7801B; or Agar Scientific, catalog number: AGL4158)
15. Grid storage box (LKB/Leica catalog number: G133, or TAAB Gilder G062)
16. Lab oven/incubator (60 °C)
17. Light microscope
18. Magnetic hotplate stirrer with magnetic stir bar
19. Metal 1.5 ml Eppendorf rack
20. Microtome (Reichert Ultracut)
21. Microwave oven
22. pH meter
23. Pipettes
24. Protective clothes and mask

25. Refrigerator (4 °C)
26. Rotary shaker or orbital shaker (IKA KS 130 Basic)
27. Slide drying rack or slide staining jar (e.g., DWK Life Sciences Wheaton)
28. Small bench clamp workshop
29. Thin painting brush
30. Transmission Electron Microscope (JEOL, model: JEM-1230)
31. Tweezers: negative-action style: thin curved tips (Dumont, catalog number: 0203-N7-PO) or thin tips (Dumont, catalog number: 0302-N0-PO-1)
32. Tweezers: straight with Geneva pattern, thin tips (Dumont, catalog number: 0103-0-PO)
33. Vortex mixer (Vortex-Genie 2)
34. Warming plate, or slide drying hotplate (Agar Scientific, model: AGL4384) or spirit lamp burner

Procedure

A. Plant material fixation

In this section, I describe the procedure for plant material preparation and fixation. This protocol was developed for *Arabidopsis thaliana* leaves, but it could also be applied to other organisms and tissues such as roots, shoots and woody tissues in tree species.

1. Sterilize the seeds before sowing
 - a. Place a small number of seeds into 1.5 ml Eppendorf tubes (approx. 5% of the Eppendorf tube volume).
 - b. Add 1 ml of 70% ethanol with Tween 20 for 2 min.
 - c. Replace 70% ethanol and Tween 20 with 1 ml of 95% ethanol for 1 min.
 - d. Remove the ethanol and wait for the seeds to dry.

Note: Perform the seeds sterilization under a sterile fume hood.

2. Grow *Arabidopsis* seedlings for 2 weeks on vertical agar plates (Recipe 1) in the chamber with long day condition (16 h) (temperature 20 °C and 18 °C at day and night, respectively).

Note: To synchronize the growth, vernalize the seeds by keeping the plates in a cold room/refrigerator at 4 °C for 2-3 days (in darkness).

3. Harvest plant material and place it directly in the vials filled with 3-5 µl of cold paraformaldehyde-glutaraldehyde (PFA-GA) fixation solution (Recipe 2).

Note: Remember to harvest the same leaf number from each plant (counting from the bottom to the top: cotyledons, leaf 1, leaf 2, leaf 3, leaf 4, meristem). To allow the fixative to penetrate well, cut small squares (~2 mm² max). in the middle part of the leaf. Harvest at least 10 leaves from different plants. Conduct all the fixation steps under the fume hood with protective gloves and clothes (for handling restrictions see Safety Data Sheet SDS provided by the retailer). Mark the vials by writing the names with a permanent marker and sticking a transparent tape on these labels.

4. Vacuum samples in a desiccator at ambient temperature until plant pieces will sink (approx. 4

h).

Note: It might happen that some of the samples are floating after vacuuming, which indicates that samples more likely contain oxygen. Try to collect samples, which are at the bottom of the vials.

5. Place the vials on a rotary or orbital shaker and leave the samples mixing for a couple of hours.
6. Keep the samples in a cold room/refrigerator (4 °C) overnight.
7. Discard PFA-GA fixative.
8. Wash the samples with phosphate buffer (PB) (Recipe 3) (twice for 30 min each).

Note: Discard PFA-GA fixative and first PB washing by using disposable plastic Pasteur pipettes in the assigned waste bottle. Wash by adding at least 5 ml of PB or distilled water (the more PB and distilled water, the better the washing). Conduct all washing steps slowly mixing on rotary or orbital shaker under a fume hood.

B. Embedding plant material in low melting point (LMP) agarose

Embedding small plant pieces in LMP agarose is the best way to orient the sample for sectioning (e.g., cross or longitudinal). It marks the direction of shoot/root tip (Figure 1A) or the localization of abaxial and adaxial leaf sides (Figure 1B). Embedding also facilitates the handling and protection of samples.

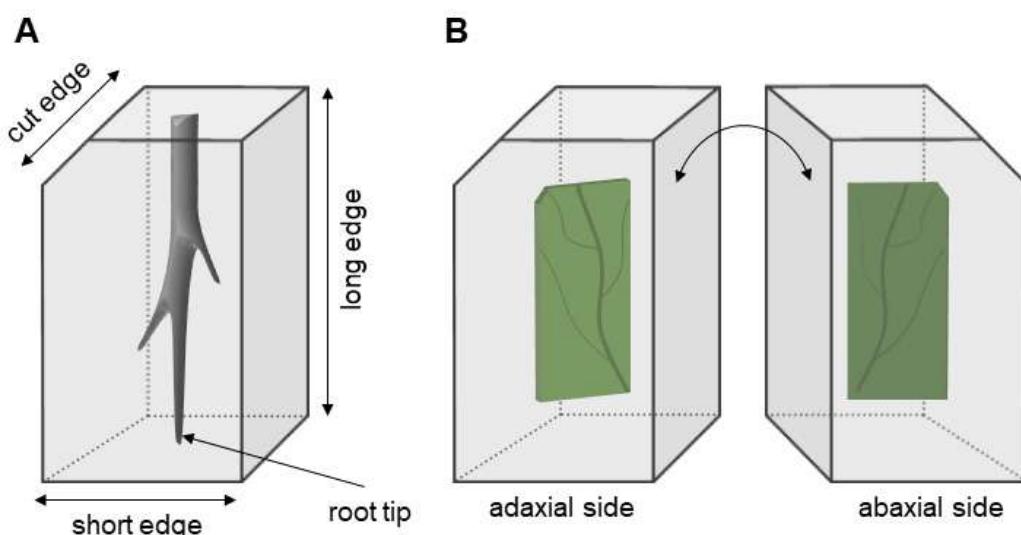


Figure 1. Orienting the plant pieces in low melting point (LMP) agarose. A-B. Samples embedded in cuboid-shaped agarose blocks presenting one cut edge to mark the orientation of the sample (e.g., top left in Figure 1A). A. Top part of the root is marked by a cut edge. B. The adaxial side of the leaf is in the front when the cut edge is on the top left.

1. Wash the samples with distilled water (twice for 30 min each).
2. Pour a thin (3-5 mm thick) layer of agarose (Recipe 4) on a square plastic Petri dish and wait a few minutes until agarose cools down (but should not solidify).

3. Place the plant material on agarose by using tweezers (many pieces on the same plate but keep distance between the samples approx. 2-3 cm²).
Note: Handle the plant specimens with care not to damage the tissues.
4. Add more agarose (\pm 3 mm thick layer) to cover the plant specimens (wait until agarose solidifies) (Figure 2A).
5. Cut off small cubes of agarose with the embedded specimens (one specimen in one cube) (Figure 2B).
- Note: The size of agarose cubes should not be bigger than the diameter of embedding capsules (8 mm Ø).*
6. Cut one of the corners in the agarose cube to orient the sample (Figures 1, 2B and 2C).
7. Move the blocks to vials filled with distilled water, then discard the water before proceeding to the next step.

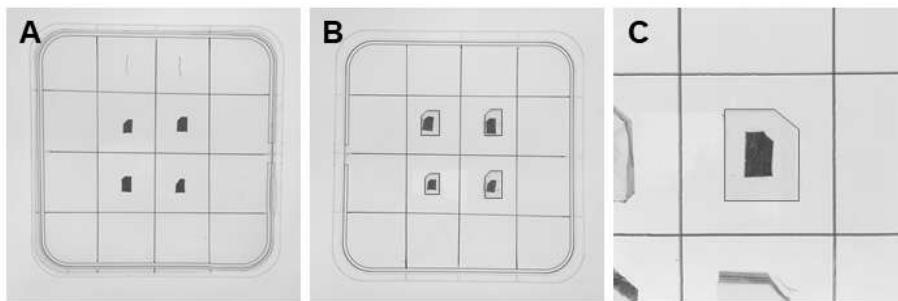


Figure 2. Embedding the plant material in agarose. A. Square Petri dish filled with a layer of 3 mm thick agarose. B. Small cuboid-shaped agarose blocks with cut one of the edges (all the leaf pieces are seen from the adaxial side). C. Magnified agar cube with the abaxial side of the leaf in the front. In this case, big plant pieces were used for visualization, but they should be \pm 5 times smaller.

C. Dehydrating specimens embedded in agarose blocks

Here I describe a common method to remove water from samples and to enable better penetration of resin. Time intervals can be adjusted; however, longer intervals give better quality embedding. Ideally, the dehydration process would be carried over the span of two days, but all dehydration steps can also be carried out within the same day (depends on the sample size).

1. Dehydrate the samples in a graded ethanol series as below (Recipe 5):
10% ethanol (twice for 30 min each)
20% ethanol (twice for 30 min each)
30% ethanol (twice for 30 min each)
50% ethanol (twice for 30 min each)
Note: The amount of ethanol added varies according to the size and number of the samples embedded in agarose. Make sure that agarose blocks are completely covered by ethanol.
2. Leave the samples in 50% ethanol in ambient temperature (on the bench) overnight.

Note: Close the vials with plastic snap-cap to prevent ethanol evaporation.

- Continue to dehydrate the samples in a graded ethanol series as below:

70% ethanol (twice for 30 min each)
80% ethanol (twice for 30 min each)
90% ethanol (twice for 30 min each)
95% ethanol (three times for 30 min each)
99.5% ethanol (three times for 30 min each)

Note: Carry all dehydration steps on rotary or orbiter shaker. The vials should be closed with plastic snap-caps. Some intermediate ethanol dilutions can be omitted (e.g., 20% and 80% ethanol), but it might affect the quality of embedding. The volume of alcohol in the last step should be the same for all vials.

D. Resin embedding

In this step, alcohol is replaced with viscous and low soluble LR white (LRW) resin. All these steps must be carried gradually with vigorous mixing of the samples on an orbital shaker. Perform all these steps in cold room/refrigerator (4 °C) to prevent resin polymerization.

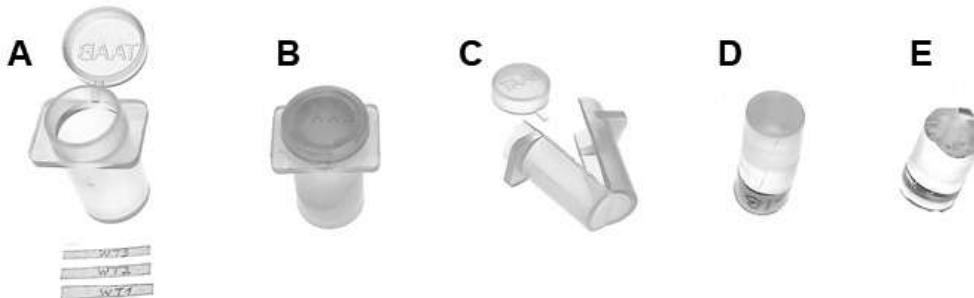


Figure 3. Embedding the plant material in resin. A. Embedding capsule and paper tags. B. Capsule after resin polymerization. C. Capsule cut along to remove the resin block. D. Resin block alone. E. Trimmed resin block into a trapezoid shape.

- Slowly add a few drops of resin to the vials with a known volume of 99.5% ethanol until the resin content will reach 10% (v/v) of the overall volume.

Note: All steps, including the exchange of the resin, must be carried out using protective gloves and clothes under the fume hood. Keep the vials closed with the plastic snap-caps mixing in the orbital shaker in cold room/refrigerator (4 °C). Always discard the resin to designated waste.

- Leave the vials in the orbital shaker to mix samples overnight.
- Exchange resin 10% in alcohol with a new resin in a graded resin series as below (Recipe 6):
10% resin (approx. 5 h)
25% resin (approx. 5 h)
50% resin overnight

75% resin (approx. 5 h)

100% resin overnight

100% (fresh) resin overnight

Note: Some intermediate resin dilutions can be omitted (e.g., 25% and 75%), but it might affect the quality of embedding.

E. Closing samples in the embedding capsules and resin polymerization

This is the last step of the embedding procedure, in which you must pay attention to properly orientate the samples (Figure 1). The agarose blocks should lie flat in the middle of the bottom part of the capsule (Figures 4A and 4B), which need to be oriented in a parallel direction towards the knife edge (see Procedure G, Figure 6). Handle the capsules with care to prevent the slipping of the samples close to the corners, which could cause some issues in trimming the sample and sectioning.

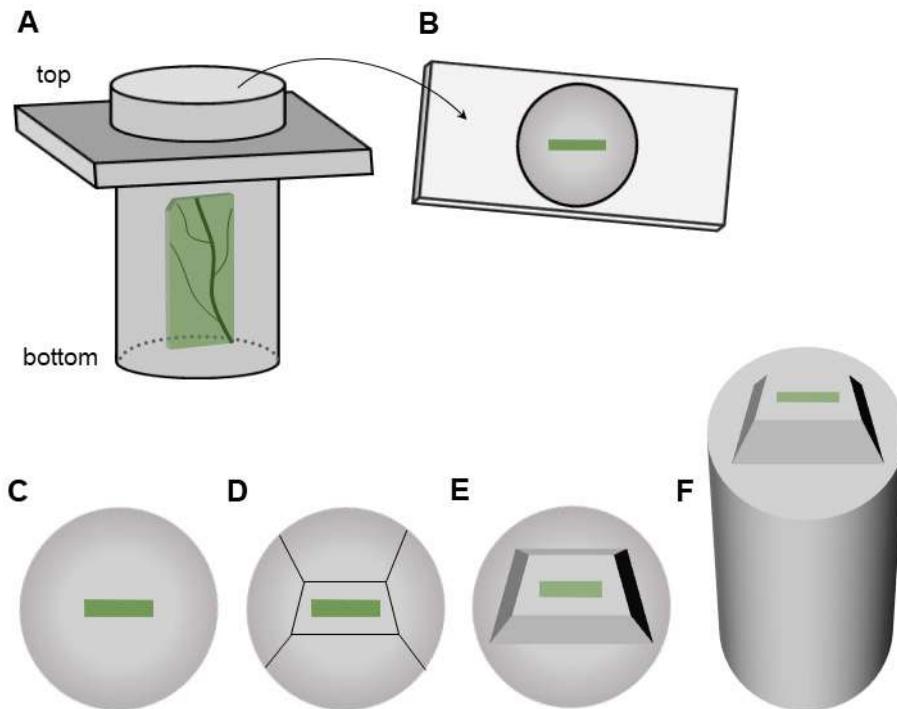


Figure 4. Trimming the plant specimen prior sectioning. A-B. A capsule with a piece of the leaf embedded in resin (A). The sample should be in the middle of the bottom part of the capsule (B). C-E. Resin with specimen alone seen from the bottom: resin before trimming (C), the direction of trimming (D), trimmed specimen (E). F. Trimmed resin block seen from the side.

1. Place the capsule (Figure 3A) on a rack and fill half of the capsule with resin.
2. Put the sample in the capsule, orient the sample well and fill-up the capsule with fresh resin.
3. Cut small paper tags, label them with a pencil and place them in the top of the capsules.

Note: The capsule should be filled with resin so that, by closing it, no space for air bubbles is left. Use a pencil to tag your samples instead of a pen or a marker, which can be washed out

by the resin.

4. Place the capsules in the incubator/oven (60 °C) for 24 h (or until it becomes solid) to polymerize the resin.

Note: Remember to check the temperature of the oven (60 °C). A temperature that is too low can affect the polymerization (resin will not polymerize equally), while a temperature that is too high or an excessively long incubation can lead to cracking or breaking of the resin.

5. Samples are ready when the resin becomes stiff (Figure 3B).

Note: To know if the resin solidified, indent a metal needle to the resin surface.

F. Preparing the grids for transmission electron microscope (TEM)

The samples for the TEM are mounted on a small grid, which is then inserted inside the TEM. The grid must be coated with formvar, which is an adhesive layer holding the sections. While preparing the grids, make sure that microscope slides, tweezers and laboratory glassware are clean (use tissue wipers and compressed air to clean the dust on the surfaces).

1. Add approx. 50 ml of formvar solution to a small beaker (Figure 5A).
2. Grab the microscope slide in a vertical position (holding a dull side) and vigorously dip approx. half of the slide in the formvar solution (no more than approx. 5 s in the solution) (Figure 5B).

Note: The formvar will form a thin film on the side.

3. Dry the slide with a film in a vertical position on a drying rack for 3-10 min.

Note: Do not touch any part of the slide, which was in contact with the formvar solution.

4. Prepare glassware with a wide diameter (≥ 10 cm) and fill it up with distilled water. Place the grids on a round silicone rubber or filter paper (Figure 5C).
5. Scratch the slide edges with a sharp razor blade and blow moist air on the slide.
6. Dip the slide vertically (90° or $\geq 45^\circ$) in the distilled water bath.

Note: You will see the film detaching from the glass and then floating on the water surface.

7. Using fine tweezers, put the grids onto a floating film with the dull side in contact with formvar (put as many grids as possible) (Figures 5D and 5E).

Note: Each grid has two sides: a dull side and a shiny side (Figure 7A). Sections will be mounted on the formvar-coated dull side (this side should be in contact with buffers, antibodies, distilled water, contrasting stain) (Figure 7B), and the shiny side will be in contact with filter paper during the drying of the samples (it is up to you which side you want to coat with formvar and put the sections on but remember to be consistent).

8. Prepare a new slide covered by one side adhesive paper or slide label (Figure 5F).
9. Grab that slide in vertical position and touch one of the edges of the floating film (Figure 5G).
10. Dip the slide into the water, which will cause adhesion of the film with grids to the slide (Figures 5H and 5I).
11. Leave the slide with grids covered with formvar until dry.

Note: Store the slides in a closed box or plastic Petri dish in cold room/refrigerator (4 °C).

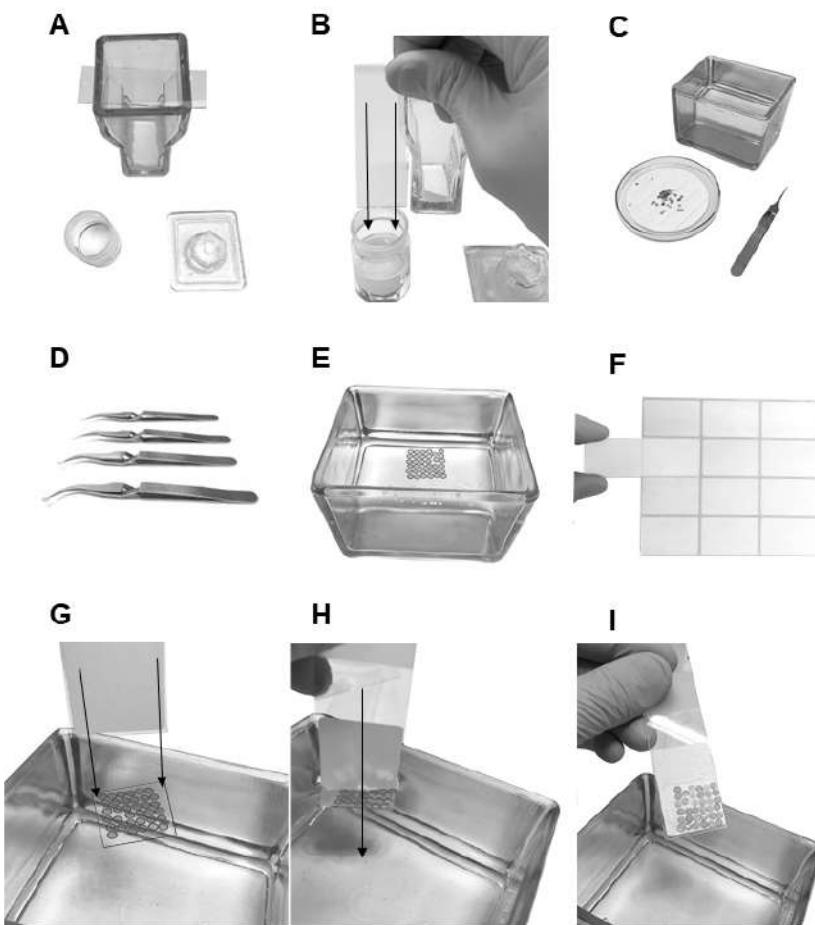


Figure 5. Preparing the grids for TEM. A. Baker filled with formvar solution and a slide on the slide staining jar. B. Dipping of the microscope slide in formvar solution. C. Glassware filled up with distilled water, Petri dish with round silicone rubber and grids on it, tweezers. D. Tweezers holding the outer ring of the grids. E. Grids on a floating formvar film with the dull side of the film. F. Sticking a slide label to the slide. G. Slide in vertically before touching the edges of the floating film. H. Dipping the slide in water causing the adhesion of film with grids to the slide. I. Slide with formvar and grids on it.

G. Ultrathin sectioning for electron microscopy

The most important thing in the preparation of the sections is to properly orient the sample. Remember to regularly control the sections quality. Make sure that the cells/tissue/organ is oriented in parallel to the knife (Figure 6) and if they are not, then correct the angle between the sample and the knife edge.

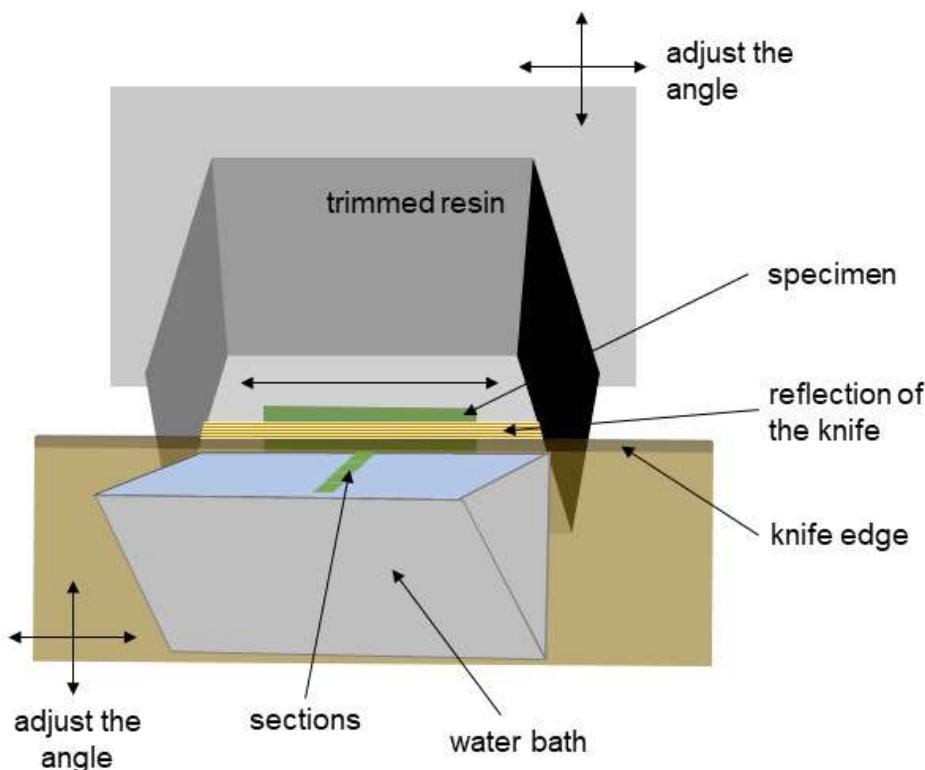


Figure 6. Ultrathin sectioning for electron microscopy. Begin with orienting the specimen in parallel to the knife edge. When resin is close to the knife, a reflection of the knife will be seen on the surface of the resin. This reflection should be parallel to the sample (if properly oriented). With sectioning progression, make sure that the cells/tissue/organ are oriented in parallel to the knife (remember to correct the angle between the sample and the knife edge).

1. Immobilize the capsules using a small bench clamp workshop.
2. Remove the capsules using stiff single edge razor blades and diagonal cutting pliers (Figures 3C and 3D).
3. Trim the bottom of the sample with a stiff single edge razor blade and then use thin double-edged razor blades for more precise cutting (Figures 3E and 4C-4F).
Note: The bottom of the sample, which will be in parallel to the knife edge (see Procedure E), should be trimmed in a trapezoid shape with the major (longer) base on the bottom and minor (shorter) base on the top (Figures 3E, 4D-4F and 6). The size of the trapezoid should not exceed the length of the diamond knife (≤ 2 mm).
4. Mount the resin block on an ultramicrotome holder with the bottom of the sample oriented towards the knife.
5. Orient the resin block with the sample in the way that the trimmed bottom of the sample (Figure 4A) will be parallel to the glass knife edge (Figure 6).
Note: When the sample is close to the knife you will be able to see the reflection of the knife on the surface of the resin (this reflection should be parallel).
6. Cut a few sections using a glass knife.

Note: The sections can be more than 1 µm thick. The purpose of this point is to see how the sections look like and to orient the specimen properly.

7. Place a drop of distilled water on a regular or a polylysine-coated slide and put a few sections on that drop.

Note: Use a wet painting brush (the thinner, the better) to collect the sections.

8. Dry sections on the slide (see Equipment 30).
9. Put a drop of toluidine blue stain (Recipe 7) onto the sections for a minute and wash under tap water.
10. Observe the sections under the light microscope.

Note: If the sample is oriented in the proper way, you can begin ultra-thin sectioning. If it is not oriented properly (cells are cut askew), adjust the sample position and repeat sectioning (Steps G5-G10).

11. Mount a diamond knife onto the ultramicrotome and fill the bath with distilled water.
12. Orient the resin block with the sample in an analogical way as above (see Step G5).
13. Begin ultrathin sectioning (70 nm thin sections) until you see the ribbon of sections floating on the water.
14. Using the forceps dip the grid under the water (the dull side with formvar on the top) and collect some sections.
15. Leave the grids with section on the filter paper or on the round silicon rubber until dry (Figure 7A, numbers 7-9), store in the grid storage box.

Note: Remember to place the shiny side on the bottom, and dull side with the section on the top.

H. Immunogold labeling

Here I describe how to perform the labeling of cell wall epitopes. In this process, primary antibodies bind to specific cell wall epitopes, which are then recognized by secondary antibodies coupled with gold particles for visualization in TEM. The immunolocalization procedure relies on moving the grids over a series of small droplets containing different solutions, antibodies and stain for contrasting, all are placed on the parafilm (Figures 7 and 8). Remember to include controls such as wild type, untreated plants as well as secondary antibody alone, which should not give any labeling.

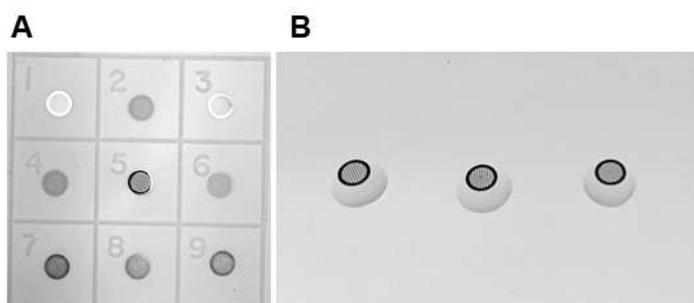


Figure 7. Grids for electron microscopy. A. Grids with two sides: a dull side and a shiny side.

Grids with the shiny side correspond to numbers 1, 3, 5. Grids with the dull side correspond to

numbers 2, 4, 6, 7-9. Sections are mounted on the formvar-coated dull side (numbers 7-9). B. The dull side is in contact with droplets. Usually, the shiny side must be in contact with the filter paper while drying the samples (not shown).

A

	BR	primary antibody	PBS	secondary antibody	distilled water
WT					
Mutant (M)					
control (WT)					
Control (M)					

parafilm

B

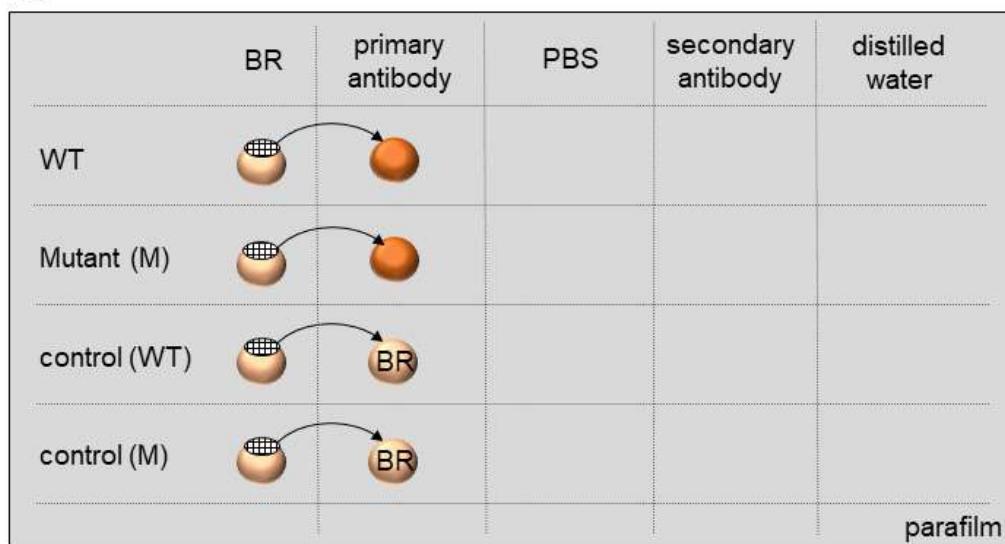


Figure 8. Immunolocalization procedure. The labeling relies on moving the grids over a series of small droplets containing different solutions (blocking reagent [BR], primary antibodies, PBS buffer, secondary antibody, distilled water) all placed on the parafilm.

1. Place a piece of parafilm on a clean table.

Note: Use 70% ethanol to clean the table. The side of parafilm covered by paper is clean and should be on top (where droplets of different solutions will be placed). Avoid touching the parafilm with hands to not contaminate it. Add a few drops of water below the parafilm and stick

the corners of the parafilm with a tape to stabilize it and keep flat.

2. Place a few droplets of BR (depending on the number of grids) in a row on the parafilm (for BR see Recipe 8) (Figure 8A).

3. Place the grids on droplets of BR and leave it for 15 min at ambient temperature (the dull side of the grid containing the section should be in contact with the droplet) (Figures 7B and 8B).

Note: Always transfer the grids with clean tweezers. It is recommended to use a set of tweezers (negative-action style). To avoid cross-contamination between antibodies, before touching the grids, always clean tweezers with 70% ethanol, rinse in distilled water and dry with a clean tissue wiper before contact with a grid. Always handle the grids with care. The tweezers should be in contact only with the external ring of the grid.

4. Put the droplets of primary antibody solutions (Recipe 9) in the second row (use BR instead of primary antibodies for the controls, which will be treated only with secondary antibodies).

5. Take the grid out of BR droplet, touch the edge of the grid with a clean filter paper (to absorb the reagent) and transfer the grid onto a droplet with primary antibody.

Note: From the circles of the filter paper, cut small triangles, use the sharp tip to touch the grid edge.

6. Incubate with primary antibody for 45-60 min at ambient temperature.

Note: During the incubation in primary and secondary antibodies make sure that the solution will not dry out. To prevent drying, cover the samples with e.g., a large glass Petri dish.

7. Put droplets of PBS (Recipe 10) in the four next rows.

8. Take the grid out of the primary antibody droplet, touch the edge of the grid with a clean filter paper (to absorb the reagent), and transfer the grid onto a droplet with PBS.

Note: Rinse the grids four times in PBS. Leave the grids for 5 min on each droplet. Before transferring to the next PBS droplet, always absorb previous PBS with a filter paper.

9. Put the droplets of the secondary antibody solution (Recipe 10) into the next row.

10. Transfer the grids in an analogical way to above (Steps H5 and H8).

11. Incubate with the corresponding secondary antibody for 60 min at ambient temperature.

12. Put the droplets of distilled water into the four next rows.

13. Transfer the grids in an analogical way to above (Steps H5 and H8).

Note: Rinse the grids four times in distilled water. Leave the grids for 5 min on each droplet. Before transferring to the next water droplet, always absorb previous water with a filter paper.

14. Take out the grid from the distilled water droplet, touch the edge of the grid with a clean filter paper (to absorb the reagent) and transfer the grid on a filter paper or on a silicon rubber (the dull side with the section should be on top).

15. Leave the grids for at least 60 min or until dry (cover with a large Petri plate to prevent contamination).

16. Put a droplet of uranyl acetate (UA) (Recipe 11) on a new piece of the parafilm.

Note: Briefly spin at maximum speed before use.

17. Transfer the grid onto a droplet of uranyl acetate (UA) and leave it for 10 min at ambient temperature in darkness (UA is light sensitive).
18. Take out the grid from UA droplet, touch the edge of the grid with a clean filter paper (to absorb the reagent) and transfer the grid onto the distilled water droplet.
19. Holding the grids with negative-action style tweezers rinse each grid under tap water for 30 s, then place the grids onto the filter paper or silicon rubber, wait until dry (the dull side with the section should be on the top) (Figure 7A, points 7-9) and store in a grid storage box.
Note: All the steps involving the use of UA must be carried with special attention by using protective gloves and clothes under the fume hood. All UA waste must be placed in assigned waste bins. Avoid too long incubation in UA as it can give too high contrast.

Recipes

1. Half strength MS basal medium
 - a. Add 2.2 g of MS
 - b. Add 10 g of sucrose (1% v/v)
 - c. Add distilled water to a final volume of 1 L
 - d. Mix the solution on a magnetic stirrer with a magnetic stir bar
 - e. Adjust pH to 5.6 by decreasing pH with hydrogen chloride (HCl) or increasing pH with sodium hydroxide (NaOH)
 - f. Add 7 g of plant agar (0.7% v/v)
 - g. Autoclave the medium
2. Paraformaldehyde-glutaraldehyde (4% PFA and 0.05% GA) fixative solution
 - a. Add 4 g of paraformaldehyde (PFA) powder to a conical flask with 100 ml of 0.1 M phosphate buffer (4% of PFA for total concentration)
 - b. Add a magnetic stir bar, cover with aluminum foil to prevent evaporation and place the flask on a magnetic hotplate stirrer (90-100 °C)
 - c. Add a few drops of sodium hydroxide solution (NaOH) to speed up dissolving the powder (use 0.1 M concentration of NaOH or lower) and then leave it mixing until the powder dissolves completely (it can take a couple of hours)
 - d. Adjust pH to 7.2 by decreasing pH with hydrogen chloride (HCl) or increasing pH with sodium hydroxide (NaOH)
 - e. Add 200 µl of 25% glutaraldehyde (GA) (0.05% of GA for total concentration)
 - f. Store the fixative solution in a designated (ventilated) refrigerator (for a short storage) or freeze the fixative in -20 °C (for long storage)

Note: PFA and GA are highly toxic. Conduct all the work under the fume hood, with protective gloves, clothes and mask. Discard the fixative into designated waste. It is recommended to prepare fresh fixative or to use already prepared PFA solution. To not generate additional waste, it is recommended to calculate the exact amount of fixative which is required (see Step A1). To

not contaminate the pH meter, it is recommended to use a disposable pH indicator paper instead.

3. Phosphate buffer (PB), 0.1 M solution (pH = 7.2)
 - a. Prepare a stock solution of 0.1 M disodium phosphate (Na_2HPO_4) and another solution of 0.1 M monosodium phosphate (NaH_2PO_4) by dissolving these salts in distilled water
 - b. Adjust phosphate buffer pH to 7.2 by mixing 68.4 ml of disodium phosphate solution (Na_2HPO_4) with 31.6 ml of monosodium phosphate solution (NaH_2PO_4) (Sambrook *et al.*, 1982)

Note: It is recommended to prepare fresh stock concentrations because salt might precipitate. Confirm the pH with a pH meter measurement. Autoclave phosphate buffer solution before storing in ambient temperature.

4. Low melting point (LMP) agarose 1% solution
 - a. Dissolve 1 g of LMP agarose in 100 ml of distilled water
 - b. Warm it up in a microwave until boiling
 - c. Wait to cool it down before contact with the plant material
5. Different ethanol concentrations 10%-95%
 - a. Mix ethanol absolute \geq 99.5% with distilled water to obtain different concentrations
 - b. Prepare around 100 ml of each concentration and keep it in closed bottles
6. Different LRW resin concentrations 10%-75%
Mix LWR resin with ethanol absolute \geq 99.5% to obtain different concentrations
7. Toluidine blue solution
 - a. Dissolve 0.5 g of toluidine blue powder in 50 ml of 70% ethanol.
 - b. Prepare 1% sodium hydroxide (NaOH) solution by dissolving 0.5 g of NaOH in 50 ml of distilled water
 - c. Mix toluidine blue solution with 1% NaOH in ratio 1:5
 - d. Adjust pH of NaOH to 2.5 by decreasing pH with hydrogen chloride (HCl) or increasing pH with sodium hydroxide (NaOH)
8. Blocking Reagent (BR) 1%
Dissolve 1 g of Bovine Serum Albumin (BSA) in 100 ml of PBS (for 1% of BR for total concentration)
9. Antibodies solutions
Primary antibodies are diluted in BR (1:10) and secondary antibodies are diluted in BR (1:50)
10. Phosphate Buffered Saline (PBS), 0.1 M solution (pH = 7.2)
8 g sodium chloride (NaCl)
0.2 g potassium chloride (KCl)
1.15 g disodium phosphate (Na_2PO_4)
0.2 g monopotassium phosphate (KH_2PO_4)
Refill with distilled water up to 1 L
Adjust pH to 7.2 by decreasing it with hydrogen chloride (HCl) or increasing it with sodium hydroxide (NaOH)

Note: Autoclave phosphate buffer solution before storing in ambient temperature.

11. Uranyl acetate solution (5%)

- a. Add 0.5 g of UA to 5 ml of double distilled water in a 15 ml tube
- b. Adjust the pH to 3.5 by decreasing it with hydrogen chloride (HCl) or increasing it with sodium hydroxide (NaOH)
- c. Fill the tube with double distilled water up to 10 ml and energetically shake the content
- d. Filter the solution through a filter paper or spin the solution in the centrifuge at maximum speed for 10 min and transfer the liquid part to a new tube
- e. Keep UA solution in the dark

Note: Prepare UA solution under the fume hood with special attention using protective gloves and clothes. All UA waste must be placed in assigned waste bins. To not contaminate the pH meter, it is recommended to use a disposable pH indicator paper instead.

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Competing interests

I declare no conflicts of interest or competing interests.

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An Adjustable Protocol to Analyze Chemical Profiles of Non-sterile Rhizosphere Soil

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[Abstract] The analysis of chemical diversity in non-sterile rhizosphere soil has been a pressing methodological challenge for years. Rhizosphere-enriched chemicals (*i.e.*, rhizochemicals) include root exudation chemicals, (microbial) breakdown products thereof, and *de novo* produced metabolites by rhizosphere-inhabiting microbes, all of which can play an important role in plant-soil interactions. The power and resolution of analytical methods and statistical analysis pipelines allow for better acquisition, separation and identification of rhizochemicals, thus providing unprecedented insight into the biochemistry underpinning plant-soil interactions. The current protocol describes a recently developed method to characterize rhizochemical profiles from plants, including crops, and is modular and customizable, allowing for application across a range of different plant-soil combinations. The protocol provides in-depth details about the experimental system for sample collection, data acquisition by liquid chromatography coupled to mass spectrometry, and analytical pipeline, which statistically selects for rhizochemicals by statistical comparison between metabolite profiles from plant-containing soil and plant-free soil. Moreover, the optional addition of chemical standards permits a semi-targeted approach, which improves the annotation of chemical signatures and identification of single rhizochemicals.

Keywords: Metabolomics, Rhizosphere, Soil chemistry, Mass spectrometry, Exudate collection, Soil leachate

[Background] Previous approaches to studying rhizosphere have often focused on sterile and hydroponic growth conditions (van Dam and Bouwmeester, 2016). These approaches limit our understanding of the multi-trophic nature of the rhizosphere, as they fail to provide information about (microbial) breakdown products of root exudation chemicals and *de novo* produced chemicals by rhizosphere-inhabiting microbes, even though these may drive plant-soil interactions in response to environmental change, such as pathogen attack or abiotic stress. The challenge arises from understanding and simplifying the complex, and often overwhelming, level of chemical diversity that originates from untargeted analyses of non-sterile soil (Figure 1). Nevertheless, untangling this diversity by identifying chemical networks, their origin and function, is critical for obtaining new insight into the

ecology of a much under-explored area of plant and soil science. Although not without its caveats, recent advances in mass spectrometry (MS) technology and statistical-analytical techniques permit powerful exploratory methods to uncover the chemistry of natural soils (Pétriacoq *et al.*, 2017; Swenson *et al.*, 2015 and 2018). Such techniques are becoming increasingly accessible.

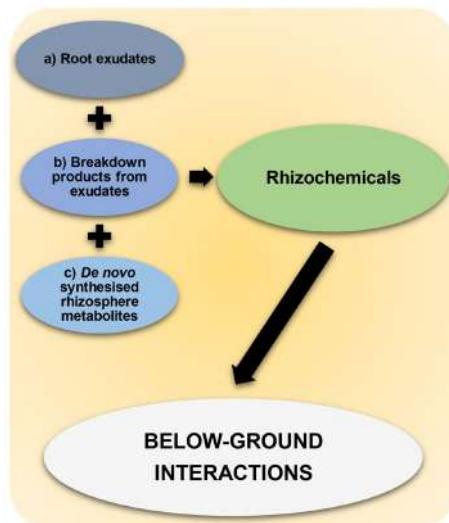


Figure 1. Rhizochemicals that shape below-ground interactions. Rhizochemicals are compounds that are enriched in the rhizosphere and include a) root exudation chemicals, b) microbial breakdown products of root exudation chemicals, c) *de novo* synthesized metabolites by rhizosphere-inhabiting microbes. Rhizochemicals can have important signaling activities thereby shaping below-ground interactions between plants and soil microbes.

The protocol presented here describes an adjustable experimental system for sample collection and sample preparation, followed by data acquisition using ultra-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF). Subsequent statistical analysis allows separating chemical signatures by retention time and mass to charge (m/z) ratios between plant-free soil and plant-containing soil, which ultimately identifies profiles of rhizosphere-enriched chemical (*i.e.*, rhizochemicals). These profiles can be further annotated using chemical databases, such as METLIN (Smith *et al.*, 2005). Moreover, during mass spectrometry examination, running a library of relevant standards can increase the accuracy of this approach and allow focus of the analysis on specific metabolite classes.

The protocol outlined here, and originally used in Pétriacoq *et al.* (2017), was initially developed for the analysis of rhizosphere chemistry in the model plant species *Arabidopsis thaliana* (*Arabidopsis*; Figures 2 and 3) and has been adapted for maize. For other plant species, a similar experimental design can be adapted and implemented, but method optimization is advised. Particularly, we recommend further optimization when insufficient separation is obtained between rhizochemical signatures from the plant-containing soil samples and the plant-free soil samples (see Figure 4). These adjustments involve *i*) testing different soils, *ii*) using different extraction solutions (*e.g.*, with varying MeOH concentrations), *iii*)

increasing root biomass in collection tubes, *iv*) adjusting soil moisture, *v*) increasing the time of sample extraction from the collection tubes, and *vi*) varying MS settings. Further attention should be paid to the fact that extraction solutions with high MeOH concentrations can cause damage to microbial and/or root cells, in which case specific assays can be used to assess the extent of cell damage, as detailed in Petriacq *et al.* (2017). If cell damage occurs, we recommend using a lower MeOH concentration in the extraction solution and/or shorter extraction times. If insufficient sample yield is obtained with shorter extraction times, a pressurized collection system can be used, as is illustrated in Figure 2D.

Materials and Reagents

Note: All chemicals and solvents used for metabolomics were of mass spectrometry grade (Sigma-Aldrich, Germany). Other solvents were of analytical grade.

1. Tips
2. Millipore miracloth, pore size of 22-25 µm (VWR, catalog number: 475855-1)
3. Petri dish (lids) Nunclon™ Delta, 8.8 cm² (Thermo Fisher Scientific, catalog number: 150318)
4. 15 ml Falcon™ conical centrifuge tubes (Thermo Fisher Scientific, catalog number: 10468502)
5. 50 ml Falcon™ conical centrifuge tubes (Thermo Fisher Scientific, catalog number: 10788561)
6. 50 ml syringe, without needle (Thermo Fisher Scientific, catalog number: 11901563)
7. 5 ml conical preparation tube (Starlab, catalog number: E1450-1100)
8. 2 ml microcentrifuge tube (Starlab, catalog number: I1420-2600)
9. 30 ml Sterilin™ universal container (Thermo Fisher Scientific, catalog number: 11309143)
10. Aluminum foil
11. Filter paper
12. 0.2 µm disposable Merck cartridge (Thermo Fisher Scientific, catalog number: 10740365)
13. 1.5 ml Glass screw neck vials, with screw caps and micro inserts (VWR, catalog numbers: 548-0018, -0020, -0106)
14. Levington M3 dry compost (Spunhill, catalog number: YEV05K)
15. Non staining silica sand (Sweepfast, catalog number: CH52)
16. *Arabidopsis* seeds
17. Maize seeds (*Zea mays*)
18. Acetonitrile (Hypergrade, Sigma-Aldrich, catalog number: 1000291000)
19. Methanol (Hypergrade, Sigma-Aldrich, catalog number: 1060351000)
20. Formic acid (FA, Sigma-Aldrich, catalog number: F0507-500ML)
21. Liquid Nitrogen (BOC)
22. Bleach Mexcel (5-10%) (SLS, catalog number: CLE0300)
23. Hydrochloric acid (37%) (Sigma-Aldrich, catalog number: H1758)
24. Sodium formate (0.01 M NaOH/1% FA (1/1, v/v) ten-fold diluted with acetonitrile/water (80/20, v/v) (Sigma-Aldrich, catalog number: 71539-500G)

25. Leucine enkephalin (2 ppm in acetonitrile/water (50/50, v/v) with 0.1% of formic acid) (Sigma-Aldrich, catalog number: L9133-25MG)
26. Ice-cold extraction solution MeOH 50% + FA 0.05% (see Recipes)

Equipment

1. Pipettes (P-5000, P-1000) (Starlab)
2. Growth tubes (make these bespoke, see below)
3. Pressure lids (make these bespoke, see below)
4. Speedvac vacuum concentrator (Thermo Fisher Scientific, catalog number: SPD120)
5. Freeze drier (Modulyo benchtop freeze dryer) (Edwards, catalog number: D-230)
6. BEH C18 column for UPLC (2.1 x 50 mm, 1.7 µm, Waters) with a guard column (VanGuard, 2.1 x 5 mm, 1.7 µm, Waters)
7. SYNAPT G2si Q-TOF (Waters)
8. Centrifuge (that fits 5 ml tubes)
9. Microcentrifuge
10. Ultrasonic cleaning bath
11. Soldering iron
12. Drill (7 mm multipurpose drill bit)
13. Growth cabinets (Fitotron, SANYO)
14. Tweezers
15. 4 °C refrigerator
16. -80 °C freezer

*Note: This equipment is described for *Arabidopsis*, but for bigger plants, such as maize, equipment use will need adjustment.*

Software

1. MassLynx, version 4.1 with Data bridge, <https://www.waters.com>
2. R with XCMS package, version 3.2, <https://www.r-project.org>
3. Multiple experiment viewer–MeV, version 4.9.0, <http://mev.tm4.org/#/welcome>
4. MetaboAnalyst version 3.0, <http://www.metaboanalyst.ca>
5. METLIN, <https://metlin.scripps.edu>

Procedure

- A. Equipment preparation
 1. Growth tubes for plant development and subsequent chemical sampling (Figure 2A).

To create growth tubes, in a fume-hood use a pre-heated soldering iron to melt 7 mm holes in the base of 30 ml plastic tubes. This step will need to be repeated for as many tubes are required. Growth tubes can be thoroughly washed with pure distilled water and reused several times. Fit tubes with 40 mm² miracloth at the bottom to hold soil. Fill tube with desired soil matrix (~40 ml), for instance 9:1 (v/v) mixture of sand and dry compost (see Materials and Reagents for more information).

Note: For larger plant species, such as 4-week-old maize seedlings, growing in clay agricultural soil, 50 ml Falcons were suitable with similar sized holes (Figure 2C).

2. Pressurized lid (for larger Falcons) to aide in pushing the liquid through the growth tube (if necessary; Figure 2D).

Create these lids by drilling a hole, smaller than the diameter of the syringe, and forcing the syringe through, securing with strong and watertight epoxy. Applying silicon to the edges and thread of the lid will ensure a tight seal to allow sufficient pressure. This set-up may be required for soil that retains more water, in order to shorten the time of flushing the soil with extraction solution.

Note: Simultaneous assays can be performed using the same experimental set-up. For instance, non-destructive rhizosphere or bulk soil can be sampled prior to extraction solution application to allow elemental analysis, or DNA/RNA extractions for determination of microbial community compositions and soil functional analysis.

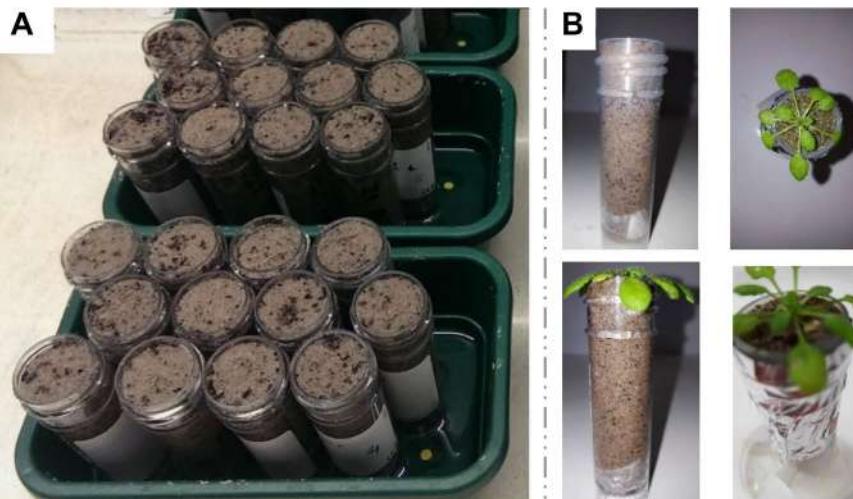
B. Experimental set-up of growth system (*Arabidopsis*; Figures 2A and 2B)

1. Stratify *Arabidopsis* seeds for 2 days in the dark in autoclaved water at 4 °C.

*Note: Seeds can be surface sterilized if appropriate for the experimental design. Such techniques are effective and straightforward (Lindsey *et al.*, 2017).*

2. Wrap soil-filled growth tubes in tin foil to limit algal growth and stand growth tubes in individual Petri dishes to prevent cross-contamination between bio-replicates.
3. Seal tubes to the Petri dish with masking tape to provide stability. Stand tubes in a tray.
4. Apply 15 ml of water to individual Petri dishes to saturate the soil.

1. *Arabidopsis thaliana*



2. *Zea mays*

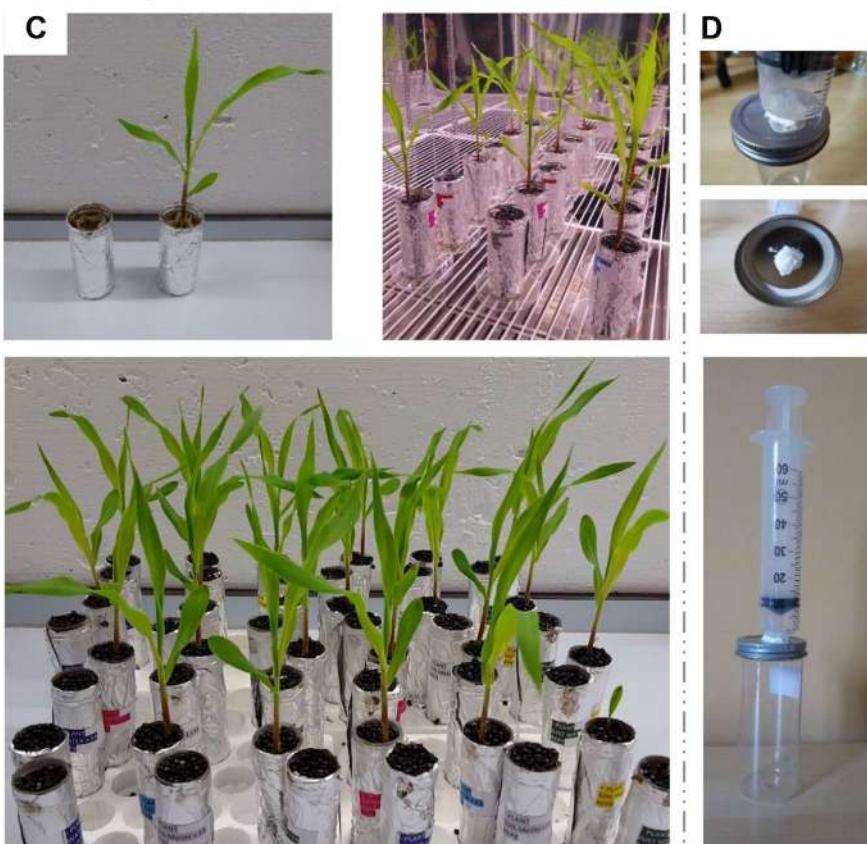


Figure 2. Experimental set-up for sampling rhizochemicals. A-C. Photos show collection tubes containing plant-free and plant-containing soils (1. *Arabidopsis* and 2. *Maize*). Tubes were covered with foil to prevent algal growth and were placed onto small Petri dishes to prevent cross-contamination. D. Example of the pressurized lid that connects to an epoxy-secured syringe. This allows the application of pressure during sample collection, in order to increase (*this word was pluralized*) the speed and yield of sample collection while minimizing exposure of living cells to

potentially damaging organic solvents. When using larger plants such as maize, the shoot is gently removed with scissors just before applying the pressure.

5. Pipette four seeds onto the soil surface of individual tubes. If desirable, prepare unseeded soil-only samples to act as controls.
6. Cover trays with a lid and place into a growth cabinet with the appropriate growth conditions.
7. Petri dishes should be supplied with 5-10 ml of water bi-weekly.
8. After seedling emergence, carefully thin seedlings using tweezers to leave one seedling per pot.

Note: In short day growth conditions (8.5:15.5 light:dark; 20 °C light, 18 °C dark; 65-70% relative humidity) final watering, prior to metabolite extraction, should be no later than 3 days.

C. Experimental set-up of growth system (Maize; Figure 2C)

1. Imbibe seeds overnight in autoclaved, sterile water before placing on Petri dishes containing sterile, damp filter paper in the dark at 23 °C for two days.
2. Plant germinated seeds in soil-filled growth tubes, 1.5 cm from the soil surface.
3. Wrap growth tubes in foil and cover the surface with black plastic beads to limit algal growth.
4. Place the tubes in a growth chamber with the desired environmental conditions.

*Note: Soil can be adapted depending on the experimental design, but more organically rich soil may produce harder to interpret results. Furthermore, depending on the soil used, 25% of perlite will aide drainage during the collection with extraction buffer (see below). For the example demonstrated here, compost was used, but agricultural soil can also be used (see Pétriacq *et al.*, 2017 for more details).*

D. Metabolite extraction from control and *Arabidopsis*/maize soil (Figure 3)

1. Collect plant soil samples from tubes containing one 5-week-old *Arabidopsis* plant, or one 17-day-old maize plant (timings can depend on experimental design).

Note: The timings used in this protocol assume similar growth conditions to those mentioned above.

2. For *Arabidopsis*, with the seedling intact, apply ice-cold extraction solution (5 ml), to the top of the tubes and avoid any disturbance of the soil surface.

Note: With larger plant, you may want to sever, or simultaneously sample the seedling. If doing so, be careful to avoid contaminating the extraction solution with damaged plant material. Furthermore, when using larger plants such as maize, the shoot can be gently removed with scissors just before applying the pressure.

3. After 1 min, 4-4.5 ml can be collected from the drainage hole in 5 ml centrifuge tubes.
4. For use with maize, gently slit the seedling with a blade around 0.5 cm of the soil surface, and subsequently apply 15 ml of the extraction solution to the soil and induce pressure to the top of the pot, using a modified lid containing a syringe.
5. After 1 min, 5-10 ml are collected in centrifuge tubes.

6. Centrifuge to pellet soil residues (5 min, 3,500 $\times g$), aliquot 4 ml of supernatant into a new centrifuge tube.
7. Flash-freeze in liquid nitrogen and freeze-dry until complete dryness.

Notes:

- a. Samples can now be stored at -80 °C until chemical analysis.
- b. If the soil is not saturated, less flow-through can be collected. The longer the buffer is in contact with the plant the more chance there is of tissue damage and bias through cellular metabolites. Limit time for which samples are exposed to MeOH is less than 1 min, to prevent the risk of root damage. Irrelevant for sampling with sterile distilled water.

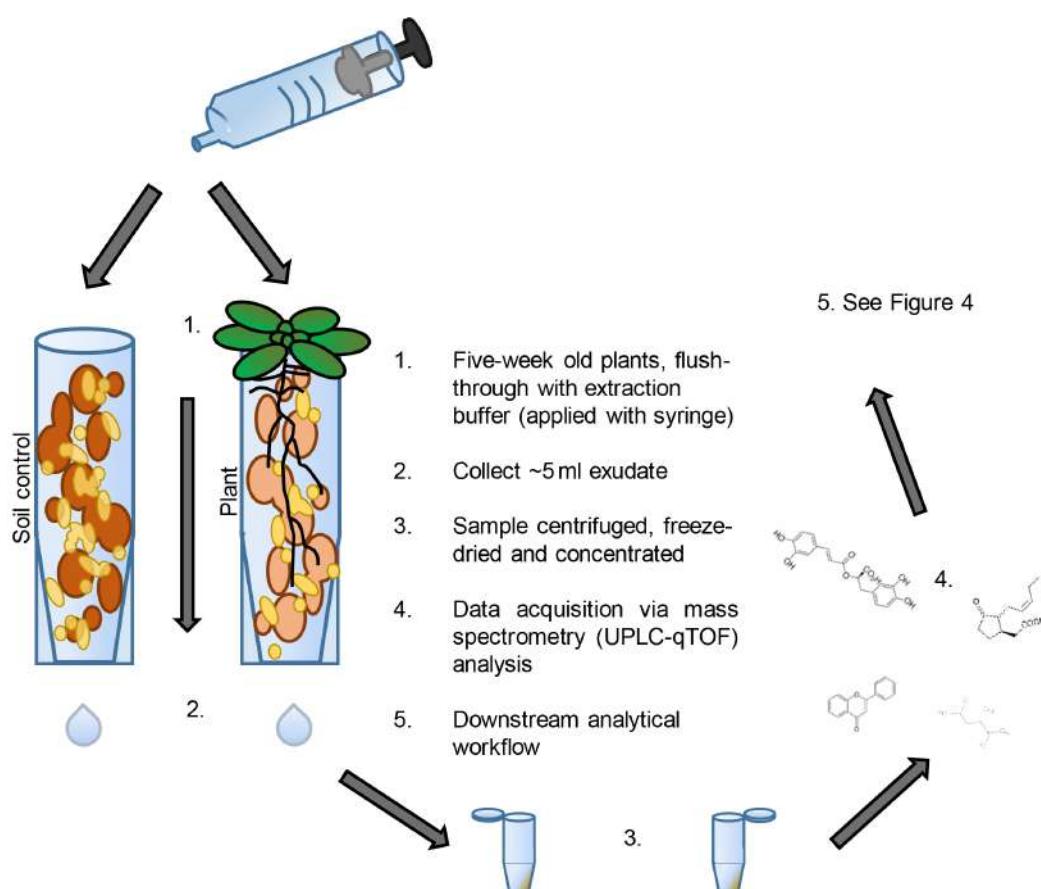


Figure 3. Sampling workflow. See figure for notes.

E. Preparing samples for UPLC-qTOF analysis

1. Resuspend dried aliquots into 100 μ l of methanol:water:formic acid (50:49.9:0.1, v/v).
2. Sonicate at 4 °C for 20 min, vortex and centrifuge (15 min, 14,000 $\times g$, 4 °C) to remove potential particles that could block the UPLC column. Alternatively, filter the samples through 0.2 μ m disposable cartridge obtained from analytical suppliers.
3. Transfer final supernatants (80 μ l) into glass vials containing a glass insert before injection through the UPLC system.

4. During this process, generate a quality control (QC) sample by mixing 5 μ l of each sample into one single vial. This will act as a control for analytical reliability of the LCMS system, and for optional normalization of the data in case of MS intensity drift when running a high number of samples (Broadhurst *et al.*, 2018).

F. UPLC-qTOF Set-up

1. LC and MS characteristics are fully detailed in Pétriacoq *et al.* (2017). Using a SYNAPT G2si HDMS Q-TOF mass spectrometer (Waters), coupled to a UPLC BEH C18 column (2.1 x 50 mm, 1.7 μ m, Waters) with a guard column (VanGuard, 2.1 x 5 mm, 1.7 μ m, Waters) for separation of compounds at a flow rate of 400 μ l min $^{-1}$, prepare the mobile phases as A; water with 0.05% formic acid, and B; acetonitrile with 0.05% formic acid with the following gradient: 0-3 min 5-35% B, 3-6 min 35-100% B, holding at 100% B for 2 min, 8-10 min, 100-5% B.
2. Set the column temperature to 45 °C and use an injection volume of 10 μ l.
3. Use a mass range of 50-1,200 Da and a scan time of 0.2 s (ESI $^-$ and ESI $^+$) with the instrument operating in sensitivity mode for the MS full scan (*i.e.*, without collision energy).
4. Ramp collision energy in the transfer cell from 5 to 45 eV (MS E), using appropriate voltage conditions for ESI $^-$ mode (*e.g.*, Capillary, -3 kV; Sampling cone, -25 V; Extraction cone, 4.5 V) and ESI $^+$ mode (*e.g.*, Capillary, +3 kV; Sampling cone, +25 V; Extraction cone, 10 V).
5. Source temperature for each mode should be 120 °C, desolvation temperature at 350 °C, desolvation gas flow at 800 L h $^{-1}$ and cone gas flow at 60 L h $^{-1}$.

G. Calibration of the qTOF mass spectrometer

1. Prior to analysis, calibrate the Q-TOF detector with a solution of sodium formate.
2. During each run, accurate mass measurements can be ensured by infusing leucine enkephalin peptide as an internal reference (*i.e.*, lock mass) with 10 s scan frequency, cone voltage of 40 V and a capillary voltage of 3 kV.
3. Inject 6 QCs samples at the beginning of the analytical run, then every 10 injections, and finish with QC sample at the end of the analytical run.
4. Inject blank samples (50% methanol, v/v) between each treatment and between ESI $^-$ and ESI $^+$ ionization modes for stabilization of the electrospray ionization source.

H. Processing of MS data for statistical analysis (see Figure 4 for a schematic of the subsequent analytical steps) and deconstruction of rhizochemicals

1. After the run has finished, convert raw files, here obtained from MassLynx, into CDF or mwXML format, using the Databridge function in MassLynx, or ProteoWizard (MsConvert).
2. For subsequent alignment and integration of metabolic peaks, use R with the XCMS package installed (Smith *et al.*, 2006) and the standard script described in [Supplemental Data S1](#).
3. Peaks can be retained for analysis when present in all bio-replicates ($n = 3$, in an example where there are 3 bio-replicates), at a threshold intensity of 10 ($I = 10$) and at maximum resolution

range of 20 ppm. XCMS parameters should be adjusted according to the mass spectrometer used in the study (here this is q-TOF), and nuances of the particular dataset being investigated.

4. Normalize peak values from each run against total ion current (TIC). For each sample, normalized peak values will generate separate datasets for ESI⁺ and ESI⁻ ionization modes.

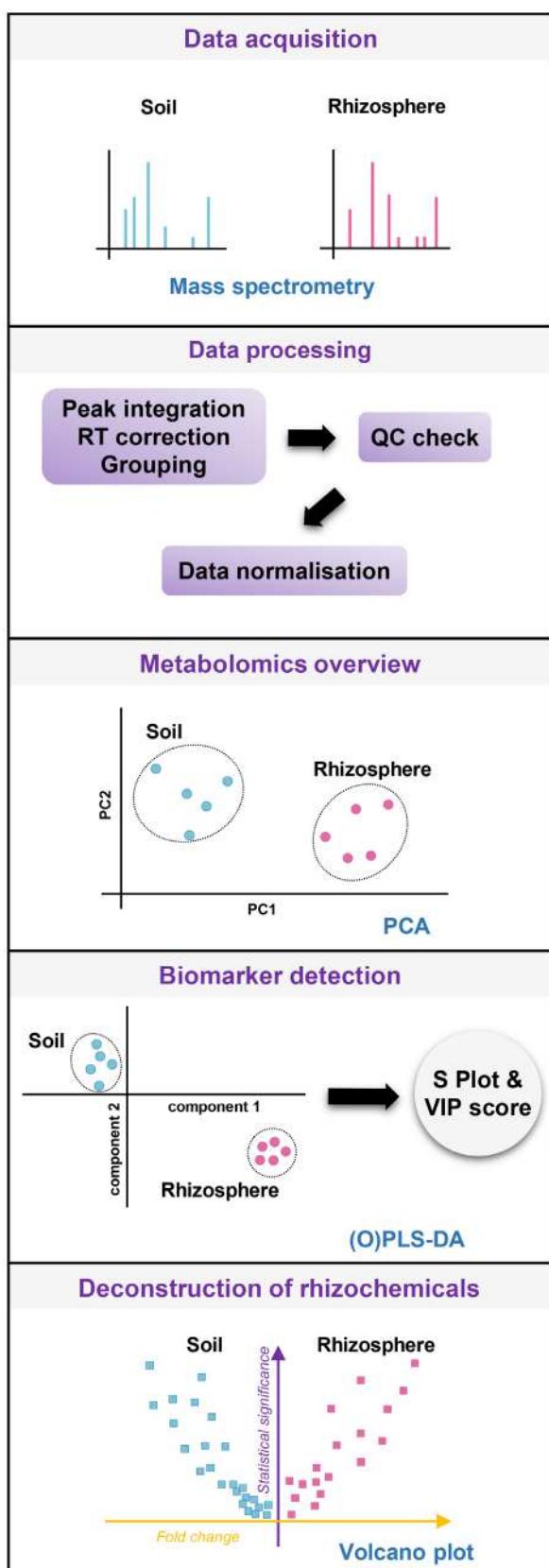


Figure 4. Analytical workflow for the identification of rhizosphere metabolites. Mass spectrometry data are processed using XCMS (Smith *et al.*, 2006) for peak identification (RT-m/z

features) and alignment, grouping, and retention time (RT) correction, then checked for quality control and normalized (Median-centered, cube-root transformation and Pareto scaling). Metabolomics overview is given by principal component analysis (PCA), which is crucial to verify sufficient metabolomics separation between extracts from plant-containing and plant-free tubes. If the global differences are not convincing by PCA, the rhizosphere chemistry in the plant-containing soil might be too much diluted by bulk soil chemistry, and further adjustments of collection systems are necessary (e.g., bigger plants, other soil, different extracting solutions, and longer sample extraction). In the specific case of binary comparison (plant-free versus plant-containing tubes), biomarker detection can be performed with S Plot obtained from (Orthogonal) Partial Least Square-Discriminant Analysis (O)PLS-DA (Worley and Powers, 2013). This supervised technique provides Variable Important for the Projection (VIP) scores that reflect the discriminant statistical power of each variable for the PLS discriminant model. Ultimately, univariate statistical comparison via volcano plots (fold change vs. statistical significance) allows for straightforward identification of metabolic markers that are specific to the rhizosphere (*i.e.*, rhizochemicals).

I. Statistical analysis of MS data

For this experimental set-up, the strength in identifying ‘rhizosphere’ chemical signatures comes from a combination of the experimental design, *i.e.*, free soil vs. planted soil, and the subsequent binary statistical comparison, obtained through a volcano plot showing statistical significance against biological significance. Further statistical tests (e.g., ANOVA, *t*-tests with consideration for multiple comparisons, such as false discovery rate–FDR or Bonferroni corrections, where appropriate) can be used for the quantification and annotation of markers that are affected by the treatment. Such pipelines depend on the conditions used in the initial structure of the experiment and question being asked.

Prior to analysis, an appropriate normalization is required. Median-normalization, cube-root transformation and Pareto scaling of the data seem to perform best on metabolomics datasets (van den Berg *et al.*, 2006): the original structure of the dataset is conserved while the influence of peaks with high intensity is softened compared to peaks with low intensity.

1. Global differences in metabolic signals between treatment/point combinations can be visualized for anions (ESI^-) and cations (ESI^+) separately, or jointly where both datasets are concatenated, by principal component analysis (PCA), using MetaboAnalyst online (v. 3.0; <http://www.metaboanalyst.ca>; (Xia *et al.*, 2015) on median-normalized, cube-root-transformed and Pareto-scaled data.
2. For quantification of the number of ions showing quantitative differences between binary treatments (specifically the comparison between plant-free soil and plant-containing soil), volcano plots can be created on median normalized, Pareto-scaled and cube-root transformed data, with a cut-off value of > 2 fold-change ($\text{Log}_2 > 1$) and a statistically significant threshold of $P < 0.05$ (Welch’s *t*-test; MetaboAnalyst). These markers can then be selected for identification. When a condition of ‘rhizosphere’ chemicals is screened against the corresponding soil, then

volcano plots can deconstruct the markers that are specific to semiochemicals of the rhizosphere. When soil chemistry is the target, conventional heatmap or cluster analysis could be performed and the relevant markers further retained for subsequent analysis (such as ANOVA with subsequent Hierarchical clustering through Pearson's correlation (Williams *et al.*, 2018). It is highly recommended that you adhere to the guidelines laid out by the Metabolomics Standards Initiative (Fiehn *et al.*, 2008; Goodacre *et al.*, 2007; Sumner *et al.*, 2007).

J. Annotation of putative metabolic markers

Putative annotation of the selected ion markers can be assigned, based on accurately detected *m/z* values at a mass accuracy < 30 ppm), using METLIN chemical database (Smith *et al.*, 2005), or other chemical/metabolite database available online, or in house. PubChem can then be used to check the predicted pathway or class annotation (<https://pubchem.ncbi.nlm.nih.gov/>).

Notes

When applying this method to larger species with greater soil volumes, it is important to note that the water content of the soil will dilute the extraction solution, which needs to be taken into account in the design of the extraction solution and the amount of flush-through collected from the sample.

In addition, the extraction solution suggested here (50% MeOH) limits tissue damage to the sample, but the actual design of this solution can be adapted depending on the chemical profile of interest. It is worth noting that both lower and higher concentrations of methanol can be appropriate. Thus, it is possible that other extraction buffers may be preferable (such as water, 70% MeOH, 10% MeOH etc.). It is highly recommended that method optimization is performed regardless, but especially in the instance of applying a different extraction solution as to those discussed.

Recipes

1. Extraction solution

50% methanol (hypergrade):49.95% ultra-purified water:0.05% Formic acid

Notes:

- a. Use clean equipment to produce this solution to the desired volume. Store in the fridge—shelf life is less than a week.
- b. Extraction solution can contain different levels of MeOH, depending on the type of chemistry that is desired for collection.

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Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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An Improved Bioassay to Study *Arabidopsis* Induced Systemic Resistance (ISR) Against Bacterial Pathogens and Insect Pests

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[Abstract] The plant immune system is essential for plants to perceive and defend against bacterial, fungal and insect pests and pathogens. Induced systemic resistance (ISR) is a systemic immune response that occurs upon root colonization by beneficial microbes. A well-studied model for ISR is the association of specific beneficial strains of *Pseudomonas* spp. with the reference plant *Arabidopsis thaliana*. Here, we describe a robust, increased throughput, bioassay to study ISR against the bacterial pathogen *Pseudomonas cannabina* pv. *alcaliphila* (formerly called *Pseudomonas syringae* pv. *maculicola*) strain ES4326 and the herbivore *Trichoplusia ni* by inoculating *Pseudomonas simiae* strain WCS417 (formerly called *Pseudomonas fluorescens* WCS417) on *Arabidopsis* plants grown in Jiffy-7® peat pellets. While most commonly used for *Pseudomonas*-triggered ISR on *Arabidopsis*, this assay is effective for diverse rhizosphere bacterial strains, plant species, pathogens and herbivores.

Keywords: Induced Systemic Resistance (ISR), *Pseudomonas simiae*, *Arabidopsis thaliana*, *Pseudomonas syringae*, *Pseudomonas cannabina*, *Trichoplusia ni*

[Background] After plant infection or colonization by pathogenic or commensal microbes, a systemic defense response can ensue involving immunological “memory” or “priming” (Parker, 2009; Fu and Dong, 2013; Martinez-Medina et al., 2016). Depending on the microbe and the associated plant organ, different systemic resistance programs are induced (Pieterse et al., 2014). When roots encounter specific commensal microbes, induced systemic resistance (ISR) in distal root and shoot tissue is observed (Pieterse et al., 1996; van Loon et al., 1998). Although the genetic and hormonal signaling mechanisms deployed during the ISR response are partially understood (Verhagen et al., 2004; Pieterse et al., 2014; Pangesti et al., 2016), many questions still remain unanswered, such as the mechanisms involved in root-microbiome communication or the identity of systemic signal(s) (Pieterse et al., 2014; Haney et al., 2018; Cecchini et al., 2019).

The inoculation of *Arabidopsis thaliana* roots with *Pseudomonas simiae* WCS417 has been used as a model system for studying ISR against bacteria, fungi and herbivore invaders (Pieterse et al., 1996;

van Loon *et al.*, 1998; Verhagen *et al.*, 2004; Haney *et al.*, 2015). Existing bioassays for *P. simiae*-*Arabidopsis* ISR make use of sterile sand-germinated *Arabidopsis* followed by seedling transplantation into a sand/soil mixture containing the rhizobacteria (Pieterse *et al.*, 1996; Pozo *et al.*, 2008; van Oosten *et al.*, 2008). Building on this method, we have recently developed ISR bioassays against the bacterial pathogen *Pseudomonas cannabina* pv. *alcaliphila* (formerly called *Pseudomonas syringae* pv. *maculicola*) strain ES4326 (*Pma*) and the herbivore *Trichoplusia ni* (*T. ni*) using *Arabidopsis* plants germinated and grown in Jiffy-7® peat pellets. This is an effective system to robustly trigger and quantify ISR, primarily because peat pellets have a constant volume and low fluorescent pseudomonad content (Haney *et al.*, 2015 and 2018; Cecchini *et al.*, 2019). Moreover, avoiding the transplantation of seedlings reduces the experimental labor and stress to the seedlings, thereby improving assay efficiency. Here, we describe a step-by-step bioassay methodology for ISR experiments that can be potentially standardized across laboratories worldwide.

Materials and Reagents

1. 96-multiwell plates (Corning, Costar, catalog number: 2797)
2. Kimwipes (Fisher, catalog number: 06-666A)
3. 15 ml and 50 ml centrifuge tubes (MidSci™, catalog numbers: 15 ml-CT2715, 50 ml-CT2750)
4. Plastic domes (Hummert International, catalog number: 11-33480) and trays (Hummert International, catalog number: 11-33010)
5. 9 cm round Petri dishes (Fisher, catalog number: FB0875712)
6. 1.5 ml microfuge tubes (MidSci™, catalog number: AVSS1700)
7. Plastic pestles for 1.5 ml microfuge tubes (Fisher, catalog number: 12-141-364)
8. 3 or 5 mm metal beads (QIAGEN, catalog number: 69997 or 69989)
9. Trays for holding the Jiffy-7® peat pellets (Hummert International, catalog number: 11311000)
10. 1 ml-syringes without needle (BD Biosciences, catalog number: 309659)
11. Mesh bags (can be made by sewing a semi-oval out of a fine washable mesh material such as bridal veil; see Figure 3 for schematic; alternatively, perforated cellophane bread bags can be used)
12. Tightly closing plastic container (with screwcap lid)
13. Damp paper towel
14. Fine bristled paint brush
15. 96-well racked collection microtubes (optional, for 96-well tissueLyser format) (QIAGEN 19560)
16. *Arabidopsis thaliana* (L. Heyhn.) ecotype Columbia (Col-0)
17. *Pseudomonas cannabina* pv. *alcaliphila* (formerly called *P. syringae* pv. *maculicola*) ES4326 (Bull *et al.*, 2010) carrying an empty vector (*PmaDG3/Pma*) (Guttman and Greenberg, 2001) bacterial culture (stored in 15% glycerol at -80 °C)
18. *Trichoplusia ni* (eggs; Benzon Research or Natural Resources Canada)
19. *Pseudomonas simiae* strain WCS417 (formerly called *Pseudomonas fluorescens* WCS417)

20. Jiffy-7® peat pellets (Jiffy products, Canada, Hummert International, catalog number: 14-23700)
21. Glycerol (Sigma-Aldrich, catalog number: G7757)
22. K₂HPO₄ (Fisher, catalog number: P288)
23. MgSO₄ (Fisher, catalog number: BP213)
24. Proteose peptone No. 3 (BD Biosciences-US, catalog number: 211693)
25. Antibiotics
 - a. Rifampicin (GoldBio, catalog number: R-120)
 - b. Kanamycin (GoldBio, catalog number: K-120)
26. Agar (Fisher, catalog number: BP1423)
27. Sterile distilled and tap water
28. Bleach (Clorox Concentrated Germicidal Bleach)
29. Ethanol (Decon Labs Inc., catalog number: 2701)
30. Triton X-100 (Fisher, catalog number: BP151)
31. Tryptone (BD Biosciences-US, catalog number: 211705)
32. Sodium chloride (NaCl)
33. Yeast extract (BD Biosciences-US, catalog number: 212750)
34. 70% ethanol (see Recipes)
35. 25% bleach supplemented with 0.1% Triton X-100 (see Recipes)
36. 0.1% agar (see Recipes)
37. King's medium B (KB) (see Recipes)
38. Luria-Bertani medium (LB) (see Recipes)
39. 10 mM MgSO₄ (see Recipes)

Equipment

1. Laboratory glassware
2. 200 µl and 1 ml, and 20-200 µl multichannel micropipettes (Gilson)
3. Forceps (Grainger, catalog number: 4CR15)
4. 1 L beaker (Pyrex, 1000)
5. Handheld electric drill (DeWALT, model: DWD110)
6. Cork borer 4 mm diameter
7. Autoclave (Primus Sterilizer Co. Inc. 1317. C.R.N: 09415.1256)
8. Microcentrifuge (Eppendorf, model: 5415D)
9. Freezer (-80 °C) (Panasonic VIP Plus, model: MDF-V76VC-PA)
10. Plant Growth chamber at 20-23 °C with 12 h light/12 h dark (~75-100 µmol s⁻¹m⁻², cool white fluorescent; A1000 Conviron Growth Chamber with *Arabidopsis* Kit, or similar. Alternatively, a growth-room with ~135-145 µmol s⁻¹m⁻² -mix 50/50 of 400-watt sodium and metal halide bulbs or 75-100 µmol s⁻¹m⁻² cool white fluorescent bulbs can be used)
11. Shaker incubator at 28 °C (Barnstead Max, model: Q 5000)

12. Balance (Mettler Toledo, model: PB1501)
13. Spectrophotometer (Bio-Mini SHIMADZU)
14. Laminar flow hood (SterilGARD 3 Advance)
15. Incubator at 28 °C (VWR, 3020)
16. Analytical balance for weighing caterpillars
17. TissueLyser Beadmill (QIAGEN, catalog number: 85300) with 24 or 96 adapters (QIAGEN, catalog number: 69982 or 69984)

Procedure

- A. Hydration of Jiffy-7® peat pellets and *Arabidopsis* seed germination and growth (Figures 1A-1C)
 1. In 1.5 ml Eppendorf tubes, surface-sterilize 100-200 *Arabidopsis* seeds by washing with 70% ethanol for 2 min followed by 5 min with 25% bleach supplemented with 0.1% Triton X-100 and three washes in sterile water. Resuspend the seeds in 1-1.5 ml of a sterile solution of 0.1% agar (prepared in water and sterilized by autoclaving) using a pipette. Alternatively, chlorine gas sterilization can be used to eliminate endophytic bacteria, particularly to avoid microbial contamination for rhizosphere microbiome studies (Haney et al., 2015).
 2. Prior to use, leave the seeds for 3-7 days at 4 °C to allow for their stratification.
 3. Prepare 8-12 Jiffy-7® pellets for each genotype per bacterial infection experiment (or 25-30 pellets per treatment for *T. ni* assays, see Data analysis) by placing them in a germination tray.
 4. Hydrate Jiffy-7® peat pellets (Jiffy Products International, Canada) by immersing the base of the pellet with tap water. Allow the pellets to stand in water for at least 1 h to allow them to hydrate.
 5. Once the pellets are hydrated, use a pair of clean/sterile forceps to gently even/level the top surface of the pellet.
 6. Sow 3-4 stratified seeds by pipetting them at the surface of each Jiffy-7® pellet and cover the trays with a plastic dome. Do not cover the seeds with soil.
 7. Transfer the trays to a growth chamber/room under 12 h day and 12 h night conditions at 23-20 °C day/night temperature regime, ~75-100 $\mu\text{mol s}^{-1}\text{m}^{-2}$ of cool white fluorescent light at rosette level, 50%-70% relative humidity. Alternatively, ~135-145 $\mu\text{mol s}^{-1}\text{m}^{-2}$ of mix 50/50 of sodium and metal halide light can be used.
Note: Intensities of cool white fluorescence > 125 $\mu\text{mol s}^{-1}\text{m}^{-2}$ cause plants to bolt between 3 and 4 weeks and so should not be used.
8. At 3-7 days post germination, carefully remove extra seedlings with clean/sterile forceps from the surface of the pellets leaving only a single seedling in each pellet (do not water seedlings during the first week after planting).
9. Two to three times per week, bottom water the Jiffy pellets by flooding the tray with tap water and pouring the excess water off after 30-60 min.

B. *P. simiae* suspension preparation and seedling/pellets inoculation (Figures 1D-1F)

1. When seedlings are eight days old, streak *P. simiae* WCS417 strain from a frozen glycerol stock onto a King's B (KB) solid medium plate supplemented with Rifampicin (100 µg/ml). Allow it to grow for 24 h at 28 °C. Pick a single colony, streak it to a new plate and grow it for another 24 h.
2. Inoculate 15 ml of media supplemented with Rifampicin (100 µg/ml) (in a 50 ml Falcon tube) with a loop of confluent bacteria from the plate. Grow in a 28 °C shaker incubator for 16-24 h.
3. Centrifuge the culture at 3,000 x g for 5 min, discard the supernatant and resuspend the pellet in 15 ml of sterile 10 mM MgSO₄ solution. Repeat the centrifugation and finally resuspend the pellet in 15 ml 10 mM MgSO₄ by pipetting up and down.
4. Make a 1/10 or 1/20 dilution of the bacterial suspension (900 µl 10 mM MgSO₄ + 100 µl bacterial suspension for 1:10 or 950 µl 10 mM MgSO₄ + 50 µl bacterial suspension for 1:20 dilution) in a 1.5 ml tube and measure optical density at 600 nm (OD₆₀₀) in a spectrophotometer to estimate the OD₆₀₀ of the original *P. simiae* culture. (Divide the measured OD by the dilution factor).
5. In a clean (non-sterile) beaker, prepare at least 1 L of the *P. simiae* WCS417 inoculum by diluting the bacterial suspension culture in 10 mM MgSO₄ to a final OD₆₀₀ of 0.01.
6. Inoculate the ten-day-old seedlings with 6 ml of the bacterial solution per plant by gently pipetting on the top surface of the Jiffy-7® pellet, near the base of the seedling, taking care to avoid contact with the leaves or hypocotyl. Then, submerge the entire pellets for ~30 s in the same solution (using the 1 L beaker with the suspension) without allowing contact of the bacteria suspension with the seedling aerial tissues. One liter of bacterial solution can support the inoculation of at least 16 pellets. For the control/mock-treated plants follow the same steps but only using sterile MgSO₄.

Alternatively: If multiple strains are inoculated onto plants within the same flat, to avoid cross-contamination of bacterial strains between pellets, 2 ml of more concentrated inoculum (OD₆₀₀ = 0.02-0.1) can be applied to the Jiffy pellets. For these assays, the pellets should be fairly dry (generally not watered after the initial soaking) and 2 days should pass after inoculation before flood watering the plants. Using this method, multiple bacterial strains and buffer-treated controls can be grown within the same flat with no detectable cross-contamination between pellets. Additionally, significantly smaller starting volumes of inoculum can be prepared.

7. After these treatments, bottom water the plants as before for an additional 15-25 days (3.5-5 week-old plants) for *Pma* assays or 20 days (4.5 week-old plants) for *T. ni* assays.

Note: Different genotypes can be planted side by side in the same tray. Rotate different trays 2-3 times per week to minimize growth variation.

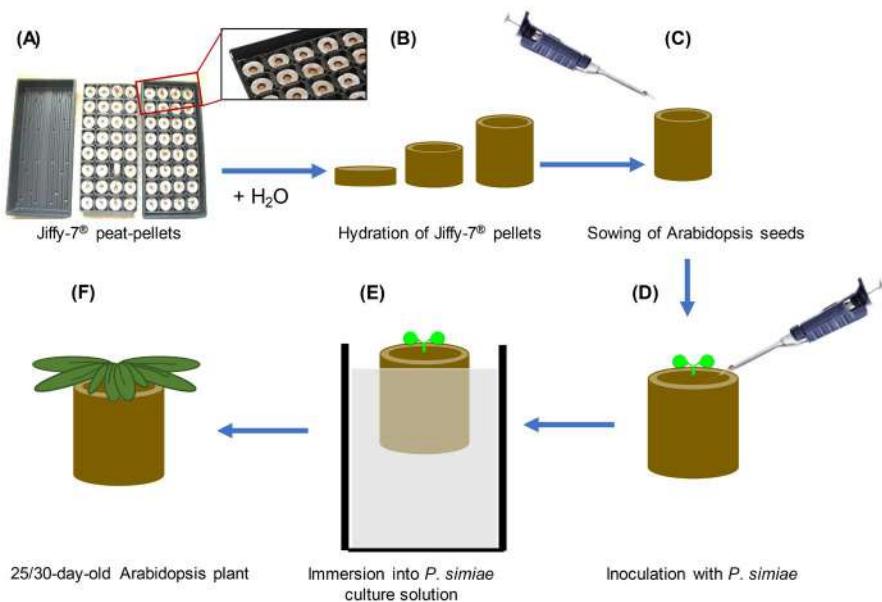


Figure 1. Schematic of *P. simiae* inoculation. A. Arrange Jiffy-7® peat pellets onto flats as shown. B. Flood the base of the trays with tap water for at least 60 min to allow the pellets to hydrate. C. Sow previously sterilized and stratified *Arabidopsis* seeds onto the surface of hydrated Jiffy® pellets. Water the pellets by flooding the base of the tray three times per week. D. Ten days after germination, inoculate 6 ml of *P. simiae* solution with OD₆₀₀ = 0.01 onto the surface of the pellet taking care to avoid contact with the seedling aerial tissues. E. Immerse for ~30 s the Jiffy® pellet into a 1 L solution of *P. simiae* of OD₆₀₀ = 0.01. One liter of bacterial solution can support inoculation of at least 16 pellets. F. Continue bottom-watering the pellets with tap water for a further 15 days (or 20 days for *T. ni* assay) until the plants are ready for plants infection with *Pma* or *T. ni*.

C. Infections and growth of *Pma* or *T. ni* to analyze the effects of *P. simiae* WCS417 ISR

To evaluate ISR against *Pma* (Figure 2)

Growing *Pma* and infection

1. Inoculate O/N cultures of *Pseudomonas cannabina* pv *alisalensis* carrying an empty vector (*Pma*) (Guttman and Greenberg, 2001) strain in KB-media supplemented with Kanamycin (50 µg/ml).
2. The next morning, dilute the cultures back in KB medium supplemented with Kanamycin (1:5 ml) and grow for additional 3-4 h.
3. Spin the bacteria down at 3,000 x g for 3 min and resuspend in sterile 10 mM MgSO₄. Repeat the wash an additional time.
4. Measure OD₆₀₀ of the suspension by diluting 1:20 as described above in section B, point 4. Dilute the culture to an OD₆₀₀ = 0.2 or 0.3 and dilute to a final OD₆₀₀ = 0.0002-0.0003 by performing three 10-fold serial dilutions in sterile 10 mM MgSO₄. The final volume should be 10 ml (~0.1 ml inoculum/leaf is required).

5. Use a 1 ml syringe without a needle pressed up against the abaxial side of the 5th and 6th *Arabidopsis* leaves to infiltrate the bacteria into the apoplast of an entire leaf. Infect at least two leaves on eight different plants. Use a felt-tipped marker to mark the petiole of infected leaves for identification leading to the sampling and quantification next steps (see below).
Note: Inoculate plants around 1 pm.
6. With a Kimwipe, dry the excess bacterial suspension from each infiltrated leaf. Let the leaves dry for 1 h until the liquid in the apoplast is no longer visible. Then, cover the plants with a plastic dome to increase the humidity and reduce the variability in bacterial growth. Return plants to the growth chamber for 2-3 days.

Quantifying bacterial growth in leaf tissues

7. Two or three days after *Pma* inoculation, excise 8 leaf discs from different plants per treatment from infected leaves (ISR-induced vs. mock) using a cork borer (4 mm diameter). Take the discs from approximately the same leaf position for all the samples, 3-5 mm from the leaf tip.
8. Using forceps, place each leaf disc in a 1.5-ml microfuge tube containing 200 μ l of 10 mM MgSO₄.
9. Grind the samples using a small hand-held electric drill with a plastic pestle. Thoroughly macerate the tissue until pieces of intact leaf tissue are no longer visible to the eye (avoiding heating up the samples by performing repeated short bursts of ~2 s instead of grinding continuously for a long-time interval). We suggest grinding each sample for a similar duration to minimize variability during the experiment. Samples can also be ground with the plastic pestle by hand (without the drill).

Alternatively: Use a bead mill or TissueLyser to homogenize the samples. To use a TissueLyser, place the leaf disk into a 2 ml Eppendorf tube with a 5 mm metal bead and 100 μ l water. Alternatively, a 96-well plate format can be used with 3 mm beads. Homogenize tissue for 2 min at 25 Hz.

10. Next, vortex the homogenate and remove 20 μ l from each sample and dilute in 180 μ l 10 mM MgSO₄ in 96-well multi-well plate. Use a multichannel pipette to repeat this process 4 times to have a serial 1:10 dilution series (10⁻¹ to 10⁻⁵) for each sample.
11. Plate 20 μ l aliquots of the 1:10 serial dilutions (10⁻² to 10⁻⁵) on KB medium plates supplemented with Kanamycin (50 μ g/ml of media) and allowed to dry onto the surface. Divide each KB plate with a marker such that at least eight samples/dilutions per plate can be plated.
Alternatively: By pouring media into rectangular plates, 10 μ l can be spotted with a multichannel pipette, and all samples can be plated on a single plate (see Figure 2).
12. Incubate the plates at 28 °C for 2-3 days until colony-forming units (CFU) can be counted. Calculate the number of CFU per leaf disc by multiplying by the corresponding dilution factor (see Data analysis).

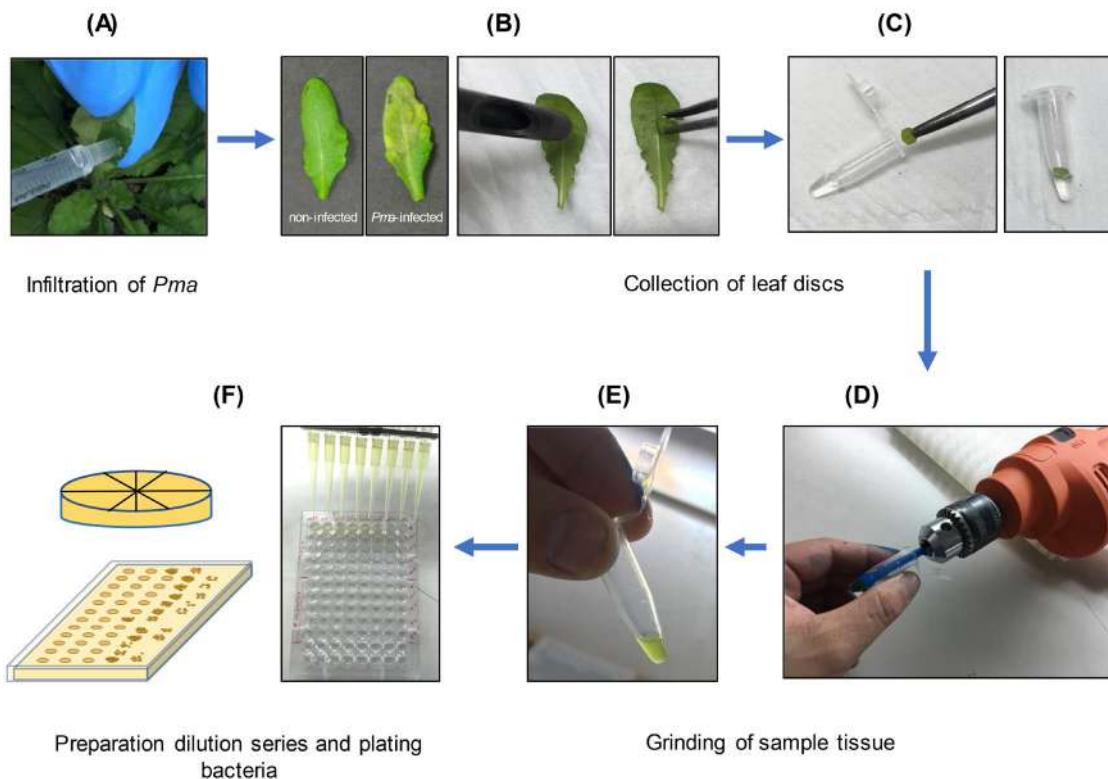


Figure 2. Images showing the key steps to evaluate ISR against *Pma* in *Arabidopsis* grown in Jiffy-7® peat pellets. A. Infiltrate the bacteria suspension into the abaxial surface of *Arabidopsis* leaves with a 1 ml-syringe without a needle (*Pma*, OD₆₀₀ = 0.0003) (Note: *Inoculate plants at ~1 p.m.*). B. Two-three days after *Pma* infection, excise leaf discs from the infiltrated leaves using a cork borer (4 mm diameter). C. Place each leaf disc in a 1.5-ml microfuge tube with 200 μ l of 10 mM MgSO₄ solution. D. Grind the samples using a plastic pestle mounted in a hand-held electric drill (shown) or a bead beater/TissueLyser (not shown). E. Grind the leaf discs until the tissue is thoroughly homogenized and no visible pieces of tissue remain (Note: *Avoid heating up the samples by performing ~2 s bursts*). F. Remove 20 μ l from each sample and dilute in 180 μ l 10 mM MgSO₄ in a multi-well plate and repeat this process to have a 1:10 dilution series (10⁻¹ to 10⁻⁵) for each sample. Plate 20 μ l aliquots of each dilution on KB medium plates or 10 μ l aliquots onto rectangular plates.

To evaluate ISR against *Trichoplusia ni* (Figure 3)

1. Incubate *T. ni* eggs (Benzon Research or Natural Resources Canada) at 30 °C for 36 h with 12-h light days. The photoperiod of the chamber should be in synch with that of the plants so the caterpillars are entrained with the same photoperiod.
2. Using a minimum of 25 plants per treatment at 4-5 weeks of age, randomly choose one newly hatched caterpillar larva from the batch. Using a small paint brush, place one larva at the center of the rosette of a Jiffy-7® pellet grown *Arabidopsis* rosette pretreated with *Pseudomonas* or buffer control.

3. Cover each pellet/plant with a mesh bag (Figure 3), return to a growth chamber, and allow the caterpillars to feed for 7 days. Plants should be at a density of no more than 30 per flat.
4. On the 7th day, remove the mesh bag and find the caterpillar on the plant. Weigh the larva with a precision balance to the nearest tenth of a milligram. The weight of newly hatched larvae is negligible; thus, the final caterpillar weight correlates with how much the caterpillar ate and host plant susceptibility (Cui et al., 2002).

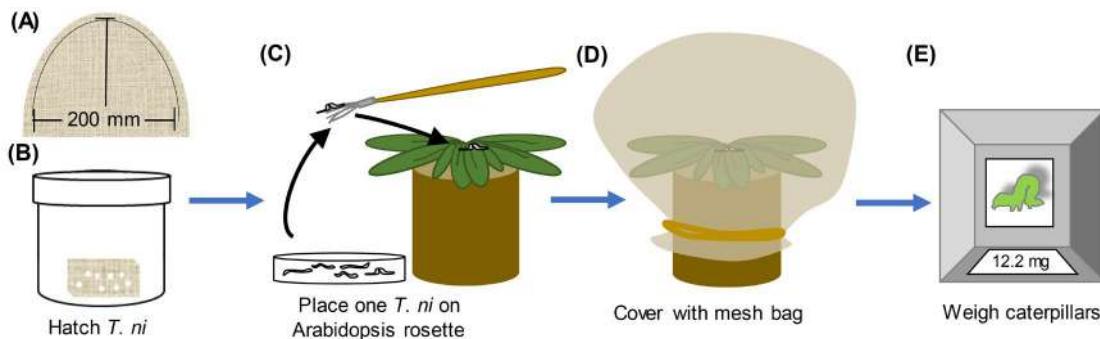


Figure 3. *T. ni* herbivory assay. A. Mesh bags can be made by cutting mesh (see materials) in a half-oval of ~200 x 200 mm and then using a sewing machine to sew two half-ovals together (dashed line). B. Place cheese cloth with *T. ni* eggs in a tightly sealing container with a damp paper towel at the bottom. Place the container in an incubator at 30 °C with the same light regime as the plants until the larvae hatch (~24–36 h). C. Using a fine paint brush, transfer a small number of newly hatch *T. ni* larvae to a Petri plate or other small container. Reseal the original container. Using the paint brush, place 1 larva on the center of the rosette of a 4–5 week-old *Arabidopsis* plant. D. Cover each plant with a mesh bag and secure at the base with a rubber band. Return the plants to the flats and return flats to the incubator. Allow caterpillars to feed for 1 week. E. One week later, find and weigh each individually (to the nearest 0.1 mg).

Data analysis

ISR-Pma data analysis

When the colonies are still small, count the CFUs for one of the dilutions (where 10–50 CFU can be counted and colonies are clearly distinct) for each disc from one leaf per plant taken and estimate the number of bacteria by multiplying with the corresponding dilution factor. Perform CFU counting for at least eight discs (12 discs provide greater statistical power and allows for increased confidence in smaller differences in bacterial growth). To increase confidence in possible differences in growth, replicate the entire experiment at least 3 independent times on different days and from different batches of inoculum. Use all the data collected from all independent experiments performed to calculate the average CFU per leaf disc plus/minus standard error. To determine if differences are statistically significant, perform analysis of variance (ANOVA) and a post-hoc test such Tukey's HSD test or Newman-Keuls (SNK) by using appropriate statistical software (Figure 4D and methods in

Cecchini *et al.*, 2019). If only two conditions are being compared, a Student's *t*-test can be used. Plot the data on a log₁₀ scale.

***ISR-T. ni* data analysis**

Weigh at least 25 larvae/plants per treatment, per experiment and repeat the experiment a minimum of 3 independent times. Use all the data collected from all independent experiments performed to calculate the average weight per larvae plus/minus standard error. To determine if differences are statistically significant, perform analysis of variance (ANOVA) and a post hoc test such Tukey's HSD test or Newman-Keuls (SNK) by using appropriate statistical software. If only two conditions are being compared, a Student's *t*-test can be used. If the experiments are done in different chambers or with insects from different sources, a significant replicate effect has been observed (Haney *et al.*, 2018). As a result, data can be normalized to the buffer-treated Col-0 control data from a single experiment prior to averaging the data from at least 3 independent experiments.

Notes

Using Jiffy-7® has allowed us to study interactions between diverse below and above ground commensals and pathogens. This setup has been used to study diverse rhizosphere bacteria that induce systemic resistance or susceptibility (Haney *et al.*, 2015 and 2018; Melnyk *et al.*, 2019). Additionally, it can be used to study below ground effects on diverse above ground pathogens including pathogenic *Pseudomonas* and *Xanthomonas* spp. (Haney *et al.*, 2018) and fungal pathogens such as *Botrytis*, and downy mildew.

Recipes

1. 70% ethanol
73.7 ml of 95% ethanol
Add distilled water up to 100 ml
2. 25% bleach supplemented with 0.1% Triton X-100
25 ml bleach and 100 µl of Triton X-100
Add distilled water to 100 ml
3. 0.1% agar
Dissolve 0.1 g agar in 100 ml sterile distilled water by autoclaving, swirl the solution while it cools
4. King's medium B (KB)
20 g of proteose peptone No. 3
10 g glycerol
1.5 g MgSO₄
1.2 g K₂HPO₄

For solid medium add 13 g agar

Add distilled water up to 1 L

Sterilize by autoclaving

5. Luria-Bertani medium (LB)

10 g tryptone

10 g sodium chloride (NaCl)

5 g yeast extract

Add distilled water up to 1 L

Sterilize by autoclaving

6. 10 mM MgSO₄

0.12 g of MgSO₄

Add distilled water to 100 ml

Sterilize by autoclaving

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Competing interests

The authors declare that they have no conflict of interests.

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Simple Method to Determine Protein Redox State in *Arabidopsis thaliana*

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[Abstract] Thiol-based redox regulation is a posttranslational protein modification that plays a key role in many biological aspects. To understand its regulatory functions, we need a method to directly assess protein redox state *in vivo*. Here we present a simple procedure to determine protein redox state in a model plant *Arabidopsis thaliana*. Our method consists of three key steps: (i) redox fixation by rapidly freezing plant tissues in the liquid nitrogen, (ii) labeling of thiol groups with the maleimide reagent, and (iii) protein detection by Western blotting. The redox state of a specific or given protein can be discriminated by the mobility change on SDS-PAGE with high sensitivity. This method provides a novel strategy to dissect the working dynamics of the redox-regulatory system in plants.

Keywords: Protein, Redox regulation, Thiol labeling, Western blotting, *Arabidopsis thaliana*

[Background] In living cells, proteins are subject to several posttranslational modifications. The dithiol/disulfide interconversion of a redox-active Cys pair, referred to as the redox regulation, is involved in a wide range of biological processes. In plant chloroplasts, this regulatory function seems to be strongly influenced by light conditions, because it is driven by a photosynthetically-generated reducing power (Buchanan, 1980). While the molecular machineries for supporting redox-regulatory system in chloroplasts, such as the thioredoxin (Trx), have been well identified so far (Buchanan *et al.*, 2002; Lemaire *et al.*, 2007; Jacquot, 2018), its working dynamics under environmental fluctuations remains poorly characterized. To address this issue, we need a methodology for analyzing the redox state of protein *in vivo*. To date, the spectrophotometric enzyme assay using a protein mixture extracted from intact chloroplasts or leaves has been often challenged on the assumption that the activation state of redox-regulated proteins is linked to their reduction level (Scheibe *et al.*, 1989; Yoshida *et al.*, 2007; Thormählen *et al.*, 2015; Vaseghi *et al.*, 2018). However, it is difficult from this assay to accurately estimate the redox state of the corresponding protein. Furthermore, we cannot exclude the possibility of nonspecific redox change during sample preparations and/or enzyme assays. To overcome these problems, an alternative approach to discriminate the redox state of proteins in a direct way is highly desirable. Here, we provide a simple protocol for determining protein redox state in a model plant *Arabidopsis thaliana*. This method has allowed us to uncover dynamic redox behaviors of several chloroplast proteins under varying light conditions (Yoshida *et al.*, 2014, 2015 and 2018; Yoshida and Hisabori, 2016a and 2018), underpinning high sensitivity and accuracy of this strategy. This method is also applicable to proteins located in other cellular compartments (Yoshida and Hisabori, 2016b).

Materials and Reagents

1. 1.5 ml microcentrifuge tube (WATSON, catalog number: 131-7155C)
2. 10 µl pipette tip (WATSON, catalog number: 110-201C)
3. 200 µl pipette tip (WATSON, catalog number: 110-705C)
4. 1,000 µl pipette tip (WATSON, catalog number: 110-804C)
5. 96-well EIA/RIA plate (Corning, catalog number: 3590)
6. Absorbent paper (ATTO, catalog number: CB-09A)
7. Immun-Blot PVDF membrane (Bio-Rad, catalog number: 1620177)
8. *Arabidopsis thaliana* plants (Ecotype: Columbia-0)
9. Liquid nitrogen
10. Distilled water
11. Tris(hydroxymethyl)aminomethane (Tris) (Nacalai Tesque, catalog number: 35434-21)
12. Sodium lauryl sulfate (SDS) (Nacalai Tesque, catalog number: 31607-65)
13. Glycerol (Nacalai Tesque, catalog number: 17018-83)
14. Protease inhibitor cocktail tablet “cOmplete” (Roche, catalog number: 11836145001)
15. Bromophenol Blue (BPB) (Wako, catalog number: 029-02912)
16. 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonate (AMS) (Invitrogen, catalog number: A485)
17. Glycine (Nacalai Tesque, catalog number: 17141-95)
18. Sodium chloride (NaCl) (Nacalai Tesque, catalog number: 31320-05)
19. Polyoxyethylene (20) sorbitan monolaurate (Wako, catalog number: 167-11515)
20. Methanol (Nacalai Tesque, catalog number: 21914-74)
21. Pierce BCA protein assay kit (Thermo Scientific, catalog number: 23225)
22. SDS-PAGE protein marker (e.g., Bio-Rad, catalog number: 161-0373)
23. ECL Prime blocking agent (GE Healthcare, catalog number: RPN418V)
24. ECL Prime Western blotting detection reagent (GE Healthcare, catalog number: RPN2232) or
ECL Select Western blotting detection reagent (GE Healthcare, catalog number: RPN2235)
25. Primary antibody for protein of interest
26. Secondary antibody for protein of interest
27. Immunoreaction enhancer (e.g., Can Get Signal, Toyobo, catalog number: NKB-101)
28. Protein extraction/thiol labeling solution (see Recipes)
29. Electrophoresis buffer for SDS-PAGE (see Recipes)
30. Transfer buffer for Western blotting (see Recipes)
31. TTBS (see Recipes)

Equipment

1. Plant growth chamber (e.g., TOMY, model: CFH-415)
2. 10 µl pipette (e.g., P10, Gilson, catalog number: F144802)

3. 20 μ l pipette (e.g., P20, Gilson, catalog number: F123600)
4. 200 μ l pipette (e.g., P200, Gilson, catalog number: F123601)
5. 1,000 μ l pipette (e.g., P1000, Gilson, catalog number: F123602)
6. Mortar (AS ONE, catalog number: 5-4054-01)
7. Pestle (AS ONE, catalog number: 5-4055-01)
8. Refrigerated microcentrifuge (e.g., TOMY, model: MX-307)
9. Heating blocks (e.g., TAITEC, model: CTU-Neo)
10. Microplate reader iMark (Bio-Rad, catalog number: 1681130JA)
11. Power supply (e.g., ATTO, model: AE-8135)
12. Equipment for SDS-PAGE (e.g., NIHON EIDO, model: NA-1012)
13. Equipment for Western blotting (e.g., BIO CRAFT, model: BE-320)
14. Luminescence image analyzer (e.g., Fujifilm, model: LAS-4000 mini)

Software

1. Software for image processing and analysis (e.g., ImageJ, <http://imagej.nih.gov/ij/>)

Procedure

An overview of the procedure is given in Figure 1.

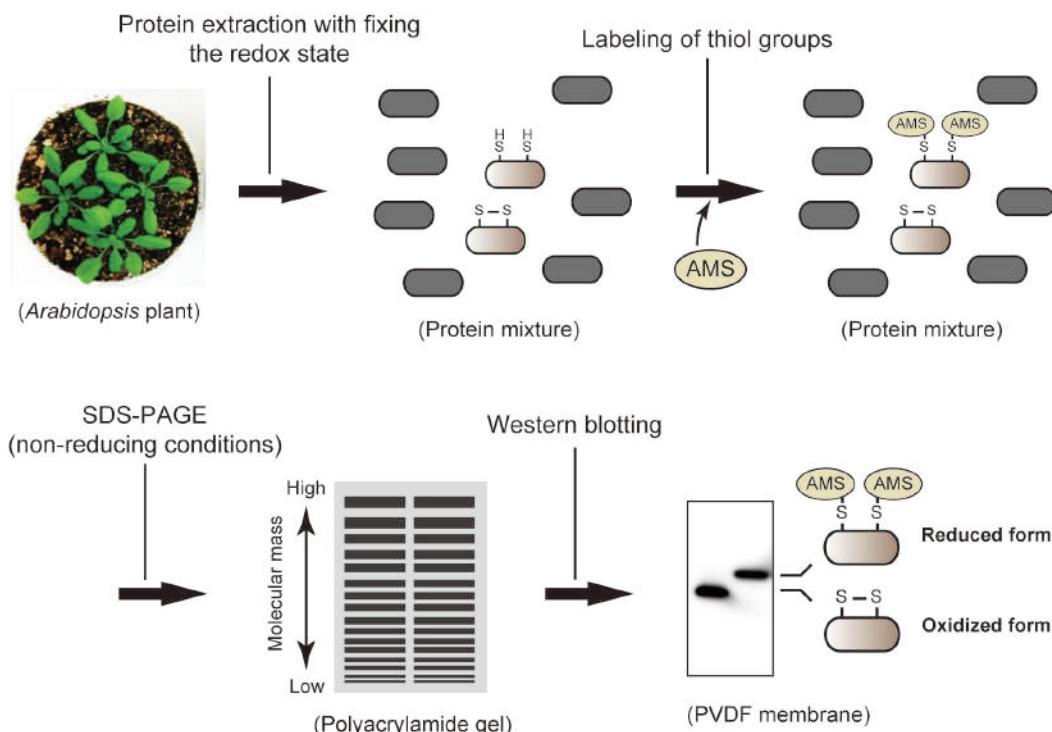


Figure 1. Simplified workflow for determining protein redox state in *Arabidopsis thaliana*

A. Flash freezing of plant tissues, protein extraction, and thiol labeling

1. Prepare the protein extraction/thiol labeling solution (see Recipes). Keep in the dark at room temperature.
2. Place *Arabidopsis* plants under intended conditions (e.g., high light conditions) for intended periods (e.g., 10 min).
3. Prepare the liquid nitrogen in a mortar just before the sampling.
4. Excise plant tissues (e.g., leaves) and immediately immerse it in the liquid nitrogen in a mortar. This step should be done under intended conditions. We usually use two or three fully expanded leaves from plants grown under long-day (16-h day/8-h night) conditions for 4 weeks (50-100 mg of fresh weight).
5. Grind plant tissues using a pestle. Transfer the powdered plant tissues to the microcentrifuge tube carefully.
6. Add the protein extraction/thiol labeling solution. For our conditions described in Step A4, more than 300 µl of the solution (3-6 µl/mg leaf fresh weight) should be added. Then, keep in the dark at room temperature for 1 h to allow free thiol groups to be fully labeled with AMS. AMS is a maleimide reagent reactive with the free thiol, but not with the disulfide bond.
7. Heat the protein sample (e.g., at 95 °C for 5 min).
8. Centrifuge at 20,000 × g, room temperature for 10 min.
9. Transfer the supernatant to a new microcentrifuge tube for removing the debris.
10. Determine the protein concentration using the Pierce BCA protein assay kit (according to the manufacturer's instructions). An equal amount of the protein extraction/thiol labeling solution should be added to the albumin standards. In our conditions described in Steps A4-A6, the protein concentration is estimated to be 4-6 mg/ml in most cases.

Note: Usually, the protein sample is stable at -30 °C for several months.

B. SDS-PAGE and Western blotting

1. Prepare the polyacrylamide gel for SDS-PAGE. We usually use the separating gel with a size of 85 x 60 x 1 mm.
- Note:* The optimal concentration of acrylamide is variable depending on the protein of interest. The molecular mass is a critical determinant. For example, we use the 10% acrylamide gel for detecting ATP synthase CF_{1-γ} subunit (ca. 35,000) or fructose-1,6-bisphosphatase (FBPase; ca. 40,000), and the 15% acrylamide gel for detecting m-type Trx (ca. 12,000) (Figure 2).
2. Set the polyacrylamide gel to the electrophoresis tank. Pour the electrophoresis buffer (see Recipes).
 3. Load the protein sample (equivalent to 10-40 µg of proteins) to each well. Run the electrophoresis.

Note: The optimal amount for sample loading is variable depending on the protein of interest. The migration time for SDS-PAGE is important for better separation of proteins in the reduced and oxidized forms, which is also variable depending on the protein of interest.

4. Prepare the PVDF membrane and absorbent papers for Western blotting. The PVDF membrane must be hydrophilically treated with the methanol (for several seconds) and then the transfer buffer (for several minutes; see Recipes) prior to the use.
5. Set the polyacrylamide gel after SDS-PAGE, the PVDF membrane, and the absorbent papers to the blotting equipment. Run the blotting.
6. After the blotting, wash the PVDF membrane with distilled water and then TTBS (see Recipes) for several minutes.
7. Incubate the PVDF membrane with 2% (w/v) blocking agent dissolved in TTBS (e.g., overnight at 4 °C).
8. Incubate the PVDF membrane with the primary antibody diluted with TTBS (e.g., at room temperature for 2 h).
9. Wash the PVDF membrane with TTBS (e.g., 10 min x 3 times).
10. Incubate the PVDF membrane with the secondary antibody diluted with TTBS (e.g., at room temperature for 1 h).
11. Wash the PVDF membrane with TTBS (e.g., 10 min x 3 times).
12. Incubate the PVDF membrane with the ECL Prime solution. Detect the luminescence.

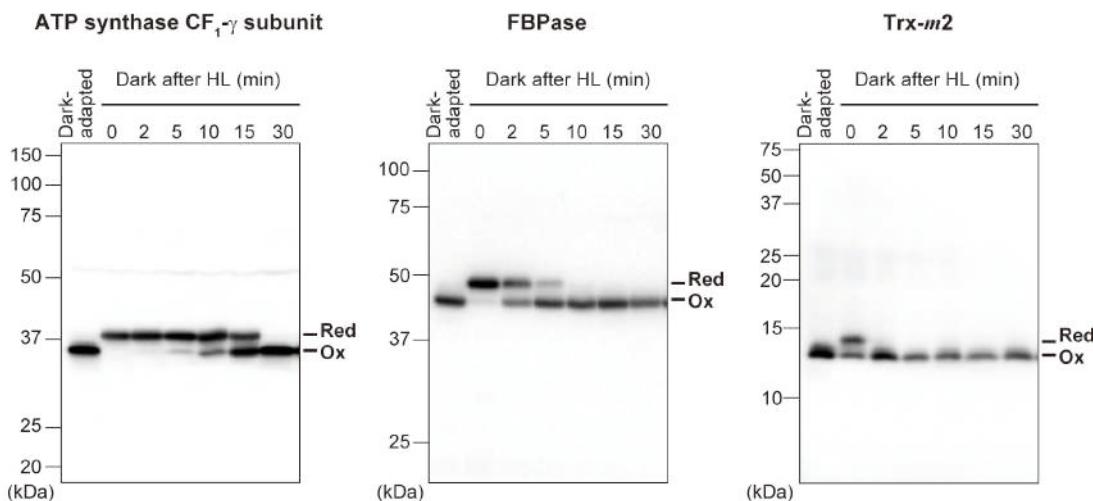
Note: The optimal conditions for the immunoreaction (Steps B6-B11) and the signal detection (Step B12) are largely variable depending on the protein of interest. The dilution ratio of antibodies should be carefully tested. If the signal is weak, it may be helpful to use the immunoreaction enhancer (e.g., Can Get Signal, Toyobo) or the high-sensitivity detection reagent (e.g., ECL Select, GE Healthcare).

Data analysis

1. The thiol-labeling maleimide reagent AMS has a molecular mass of 536.44 and, thereby, lowers protein mobility on SDS-PAGE. We can discriminate the redox state of objective protein based on an observable band shift (Figure 1). The scale of band shift is apparently variable depending on the protein of interest. If necessary, the reduction level of protein can be calculated by quantifying the signal intensity from the reduced and oxidized forms (using the image-analyzing software such as ImageJ).
2. By applying this method to protein samples sequentially collected from changing environmental conditions (e.g., light), we can chase the redox behaviors of proteins under environmental fluctuations (Figure 2). Furthermore, a combined dataset for multiple proteins can provide insights into the working dynamics of the overall redox-based regulatory network *in vivo* (e.g., Yoshida *et al.*, 2018).
3. A constitutively expressed protein should be analyzed as the loading control. We usually analyze the Rubisco large subunit.
4. Besides the formation of disulfide bond, some proteins undergo another type of oxidation, including sulfenylation and nitrosylation. To discriminate them, we need additional approaches

such as the mass spectrometry-based analysis.

A: HL to dark transitions



B: Dark to HL transitions

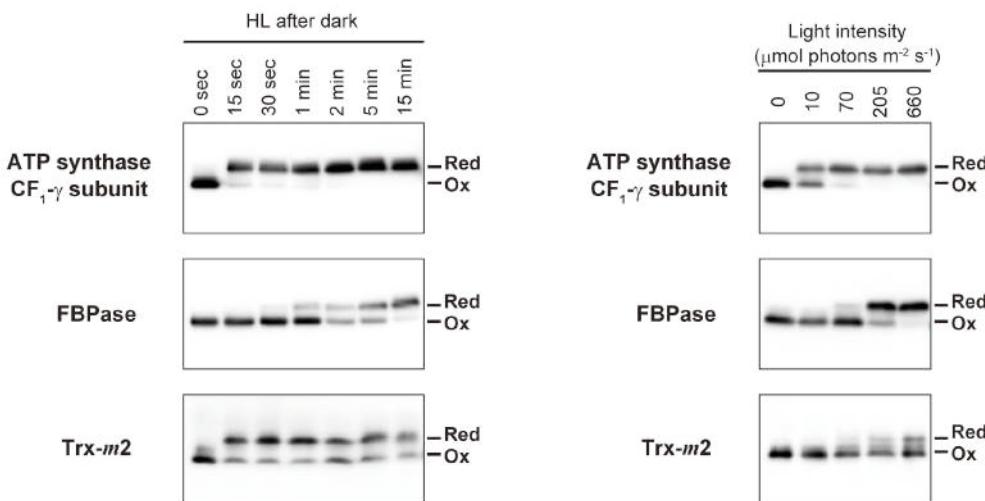


Figure 2. Examples for protein redox dynamics revealed by this protocol. Results of three chloroplast proteins (ATP synthase CF₁- γ subunit, fructose-1,6-bisphosphatase (FBPase), and *m*-type Trx (Trx-*m*2)) are shown. The protein samples were prepared from *Arabidopsis* plants placed under high light (HL) to dark transitions (A), under dark to HL transitions (B), and at different light intensities for 15 min (C). Similar results are shown in our previous papers (A: Yoshida *et al.*, 2018; B: Yoshida and Hisabori, 2018; C: Yoshida *et al.*; 2015). See each of these original papers for the detailed sampling conditions.

Recipes

1. Protein extraction/thiol labeling solution

62.5 mM Tris-HCl (pH 6.8)

2% (w/v) SDS

7.5% (v/v) glycerol

0.01% (w/v) BPB

4 mM AMS

Note: AMS must be added just before the use. Protease inhibitor cocktail tablet “cOmplete” should be also added. Dissolve one tablet in 2 ml distilled water, which can be used as 25x stock solution.

2. Electrophoresis buffer for SDS-PAGE

25 mM Tris

0.1% (w/v) SDS

192 mM glycine

3. Transfer buffer for Western blotting

25 mM Tris

192 mM glycine

20% (v/v) methanol

4. TTBS

20 mM Tris-HCl (pH 7.5)

500 mM NaCl

0.1% (v/v) polyoxyethylene(20) sorbitan monolaurate

Acknowledgments

This protocol was adapted from Yoshida *et al.* (2018) and our other studies (see Background). This study was supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI (Grant numbers: 26840090 to K.Y., 16H06556 to K.Y. and T.H.).

Competing interests

The authors declare that there is no conflict of interest.

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Transient Expression Assay in Strawberry Fruits

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[Abstract] Strawberry, including the woodland strawberry *Fragaria vesca* (2x) and the cultivated strawberry (*Fragaria × ananassa*, 8x), has emerged as a model system for studying fruit development and ripening. Transient expression provides a quick assay for gene functions or gene interactions. In strawberry, virus-induced gene silencing (VIGS) and *Agrobacterium tumefaciens*-mediated transformation in fruit have been widely used as the transient expression approaches. Unlike VIGS, the latter one can be utilized not only for gene knock-down, but also for overexpression and knock-out. Here, we show the procedures of transiently expressing the 35S::*FveMYB10* construct into fruit of the white-fruited *F. vesca* accession Yellow Wonder. As a master regulator of anthocyanin production, overexpressing *FveMYB10* will cause fruit coloration, which was observed at one week post infiltration. We also exhibit the previous results of knocking down *Reduced Anthocyanin in Petioles* (*RAP*), encoding an anthocyanin transporter, by RNAi in fruit of the strawberry cultivar ‘Sweet Charlie’. Overall, *Agrobacterium*-mediated transient transformation in strawberry fruit is a quick and versatile approach for studying gene functions in fruit ripening.

Keywords: Transient expression, *FveMYB10*, Overexpression, *RAP*, RNAi, *Agrobacterium*, Strawberry

[Background] Cultivated strawberry (*Fragaria × ananassa*, 8x) is an economically important fruit crop grown worldwide with lovely appearance and rich nutrition. The wild diploid strawberry *Fragaria vesca* has emerged as a model plant for cultivated strawberry as well as other fleshy fruit species. Moreover, strawberry is a typical non-climacteric fruit, studies on the ripening of which lack a nice model system, like tomato as a model of the climacteric fruit. Therefore, strawberry is also frequently used for studying fruit ripening.

Stable transformation is doable in both woodland and cultivated strawberry; however, the entire process is labor-intensive and time-consuming. In contrast, the transient expression assay in fruit is much faster and more efficient. There are two transient gene expression approaches in strawberry, namely Virus-Induced Gene Silencing (VIGS) and *Agrobacterium*-mediated transformation (Spolaore et al., 2001). Some studies obtained nice results by using VIGS (Jia et al., 2011; Li et al., 2019). However, we utilize the *Agrobacterium*-mediated transformation more frequently as it can achieve more experimental aims, including gene knock-down (Hoffmann et al., 2006), overexpression (Huang et al., 2018), and knock-out (Tang et al., 2018).

The *F. vesca* fruit takes 25-30 days from being pollinated to ripen under our growth conditions. The entire process is divided into seven stages: flower/anthesis, small green, medium green, large green,

white, turning, and red. Fruit receptacle (the juicy flesh) at the large green stage, when the firmness starts to decline rapidly (before the achenes turn red for the red strawberry fruit), is suitable for agrobacterium injection. Fruit receptacles at prior stages are recalcitrant to the injection owing to the texture. Thus, *Agrobacterium*-mediated transformation in strawberry fruit delimits functional studies on genes acting at late developmental stages.

The bright red color of strawberry fruit is caused by the accumulation of anthocyanin compounds, which is a visible phenotype to check. In strawberry, the R2R3-MYB transcription factor *FveMYB10* is a master regulator of anthocyanin synthesis (Lin-Wang et al., 2014). The woodland strawberry includes both red-fruited and white-fruited varieties, owing to a natural SNP occurred in *FveMYB10*; overexpression of *FveMYB10* results in accumulation of red pigments in fruit of the white-fruited accession Yellow wonder (YW) (Hawkins et al., 2016). Recently, we identified one anthocyanin transporter encoding gene *Reduced Anthocyanin in Petioles* (*RAP*) through chemical mutagenesis, knock-down of which greatly reduced fruit pigmentation (Luo et al., 2018).

In this study, as an example, we describe the procedures of transiently overexpressing *FveMYB10* in fruit of YW. Moreover, we exhibit the results of knocking down *RAP* in fruit of the strawberry cultivar 'Sweet Charlie'.

Materials and Reagents

1. Tips
2. Tag
3. 1 ml syringe with needle (GEMTIER, catalog number: 0.45X16 RW LB)
4. The white-fruited *F. vesca* accession Yellow Wonder (YW), and the strawberry cultivar 'Sweet Charlie'
5. Binary vectors: pK7WG2D for overexpression and pK7WIWG2D for RNAi. Both vectors contain a 35S::GFP cassette as a visual reporter
6. Target genes: *FveMYB10*, FvH4_1g22020/gene31413; *RAP*, FvH4_1g27460/gene31672
7. Yeast extract (OXOID, catalog number: LP0021)
8. Tryptone (OXOID, catalog number: LP0042)
9. Agar (TSINGKE, catalog number: 1182GR500)
10. MS (PhytoTechnology Laboratories, catalog number: M404-50L)
11. GV3101 Chemically Competent Cell (Shanghai Weidi Biotechnology, catalog number: AC1001)
12. NaCl (HUSHI, catalog number: 10019318, CAS: 7647-14-5)
13. Sucrose (HUSHI, catalog number: 10021418, CAS: 57-50-1)
14. Antibiotics, including Kanamycin (Kan), Gentamicin (Gent), Rifampicin (Rif), and Spectinomycin (Spe) (Biofroxx, catalog numbers: 1162GR005, 1463GR001, R3501, and S8040, respectively)
15. Luria Broth (LB) medium (see Recipes)
16. Injection buffer (see Recipes)
17. Antibiotics (see Recipes)

Equipment

1. Incubator used for the growth of *Agrobacterium* at 28 °C (JINGHONG, catalog number: DNP-9022)
2. Double temperature controlled thermostat (MIULAB, catalog number: BTH-100)
3. Centrifuge (Eppendorf, 5424)
4. Clean workbench (AIRTECH, SW-CJ-1FD)
5. Full temperature shaker (Peiying, catalog number: THZ-C-1)
6. Ultra-low temperature refrigerator (ThermoFisher SCIENTIFIC, Thermo Scientific™ Forma™ 88000)
7. Fluorescence dissecting stereomicroscope (Leica, catalog number: M205FA)
8. Plant growth room: 25 ± 3 °C, 16 h light/8 h dark, light intensity at 100 μmol m⁻² sec⁻¹

Procedure

1. Grow strawberry plants in the growth room for 3 to 4 months until the flowers open.
2. Pollinate the flowers manually; wait until the fruits develop to the large green stage, at about 15–20 days post pollination when the receptacle starts to turn soft.
3. Transform the 35S::*FveMYB10* construct (binary vector pK7WG2D) into the *Agrobacterium tumefaciens* strain GV3101, or use the *Agrobacterium* stock stored in the ultra-low temperature refrigerator. Pick a single positive colony and put into 2 ml of the liquid LB medium with the appropriate selective antibiotics, in this case, Gent + Rif + Spec.
4. Shake the culture overnight (~12-16 h) at 28 °C at the rate of 220 rpm.
5. (Optional) Amplify the fragments in the vector by regular PCR to make sure that the culture is correct.
6. Spin down the culture at 2,500 x g for 5 min, discard the supernatant. Suspend the culture with the injection buffer (MS salt + 2% sucrose) to reach a final OD₆₀₀ of 0.8.

Note: One milliliter cell suspension is enough for injecting 5-8 fruits. Two milliliters of culture usually makes 4 ml of cell suspension. The volume of the Agrobacterium culture could be increased if more injections are required.

7. Use a 1 ml hypodermic syringe to do the injection immediately. Suck up the solution into the syringe, insert the needle tip into the center of the fruit from the apex, and gently press the syringe to release the solution. For a big fruit, such as that of the cultivated strawberry, more injections in other parts of the fruit are sometimes required to make the entire fruit soaked with the solution (Video 1).

Note: Inject at least 10 fruits for each construct. Use fruits infiltrated only with the injection buffer as the negative control.



Video 1. Procedure for the fruit injection as stated in Step 7

8. Write down the construct name and the date on the tag, and tie the tag around the fruit stem. Put the plants back to the growth room, and let the fruit grow for about one week.
Note: Fruit coloration may start to show at about 3 days post injection.
9. Examine the fruit color and the GFP signal (if necessary) using the fluorescence dissecting stereomicroscope. Take pictures or collect tissues for downstream analysis.
Note: Cut the fruit in order to observe the internal phenotypes. If necessary, the GFP fluorescence can be used as a guide for collecting tissues for downstream analysis.

Data analysis

Fruits of the *F. vesca* accession YW at the large green stage (15-20 days post pollination) were used for transiently overexpressing *FveMYB10* (Figure 1A). We can see that the injected fruits are full of water under the receptacle skin (Figure 1B). One week later, fruit coloration has been fully developed (Figure 1C). Frequently, some area of the receptacle turns red, while the rest remains white. When the fruit is cut into two halves, tissues close to the skin turn red, while the inner part remains white. The intensity of the GFP fluorescence correlates well with the fruit coloration (data not shown). In contrast, fruits injected with only the injection buffer (negative control) stay white (Figure 1D). Anthocyanin synthesis or ripening genes are often transiently modified in cultivated strawberry. In order to illustrate that this approach is also suitable for gene knock-down, we exhibit the cultivated strawberry fruit transiently transformed with the *RAP-RNAi* construct (binary vector pK7WIWG2D) (Figure 1E) (Luo et al., 2018). Of note, the parts with GFP fluorescence overlap with the parts possessing color change, although *GFP* and *RAP* are independently driven by the 35S constitutive promoter in one construct.

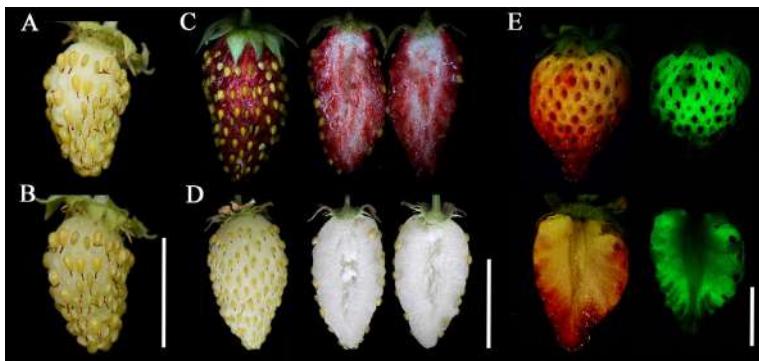


Figure 1. Phenotypes of the transiently transformed strawberry fruits. A. One fruit of the *F. vesca* accession YW at the large green stage. B. The same fruit from A that was just injected. C. One YW fruit showing red color after one week of injecting the *FveMYB10-ox* construct. D. The control YW fruit injected only with the injection buffer. E. One fruit of the strawberry cultivar ‘Sweet Charlie’ showing reduced anthocyanin accumulation after one week of injecting the *RAP-RNAi* construct. Right images showing the GFP signal taken by the fluorescence microscope. Scale bars = 1 cm.

Notes

1. Due to the limitation of fruit developmental stages used for injection, *Agrobacterium*-mediated transformation is more suitable for studying genes modulating late developmental processes, such as fruit ripening and the formation of fruit quality (coloration, formation of aroma and taste, etc.).
2. The transformation efficiency varies greatly among individual fruit, even within one fruit. We suggest using vectors carrying a visible reporter to facilitate phenotype observation and tissue collection. GFP or other fluorescent proteins are better options than the β -glucuronidase (GUS).
3. Grow the plants at about 25 °C. Lower or higher temperatures (30 °C) will greatly reduce the efficiency or even lead to a complete failure.
4. We succeeded in transforming a mixture of agrobacteria harboring two constructs, respectively, in a ratio of 1:1. This provides more potentials of examining the interactions between two or more genes.
5. Besides *F. vesca* and *F. × ananassa*, this approach should be applicable to other *Fragaria* species as well.
6. In this transient strawberry transformation, no acetosyringone is used. In the strawberry stable transformation, acetosyringone is added into the incubation buffer, and a few hours’ incubation of the cell suspension is required. This optimization might be tested in the future.

Recipes

1. Luria Broth (LB) medium
10 g L⁻¹ Tryptone

- 5 g L⁻¹ Yeast extract
10 g L⁻¹ NaCl
pH 7.0
15 g L⁻¹ Agar (for the solid LB medium)
2. Injection buffer
- 0.44 g MS powder
2 g Sucrose
100 ml ddH₂O
pH 5.8
3. Antibiotics
- 50 mg L⁻¹ Gent
50 mg L⁻¹ Rif
50 mg L⁻¹ Spe

Acknowledgments

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Competing interests

All the authors declare that they have no competing interests.

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Quantification of Blumenol Derivatives as Leaf Biomarkers for Plant-AMF Association

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[Abstract] Symbiotic interactions between arbuscular mycorrhizal fungi (AMF) and plants are widespread among land plants and can be beneficial for both partners. The plant is provided with mineral nutrients such as nitrogen and phosphorous, whereas it provides carbon resources for the fungus in return. Due to the large economic and environmental impact, efficient characterization methods are required to monitor and quantify plant-AMF colonization. Existing methods, based on destructive sampling and elaborate root tissue analysis, are of limited value for high-throughput (HTP) screening. Here we describe a detailed protocol for the HTP quantification of blumenol derivatives in leaves by a simple extraction procedure and sensitive liquid chromatography mass spectrometry (LC/MS) analysis as accurate proxies of root AMF-associations in both model plants and economically relevant crops.

Keywords: Arbuscular mycorrhizal fungi, AMF, Fungus-plant symbiosis, Leaf biomarker, Shoot biomarker, Blumenol derivatives, UHPLC-MS/MS technique

[Background] The widespread mutualistic relationship between AMF and plants involves not only the beneficial exchange of nutrients between the involved partners; phosphorous and nitrogen are supplied by the fungus and carbon is supplied by the plant in exchange; but is also thought to regulate plant growth and tolerance to various biotic and abiotic stresses (Maier et al., 1995; Barin et al., 2013; Aliferis et al., 2015; Wang et al., 2018). These interactions have fueled vast research programs and, in conjunction with dwindling natural phosphorous supplies, are of high interest for sustainable agriculture (Basu et al., 2018). Until now, the available approaches to measure and quantify AMF-plant associations require excavation of the roots followed by microscopic analysis, transcript analysis or quantification of fungal fatty acids (Barin et al., 2013). However, these methods are impractical for HTP screening due to the damage that results from root sampling, as well as being laborious (Barin et al., 2013; Wang et al., 2018). Hence, an HTP screening technique is needed to empower research and development in breeding programs for improved AMF-plant associations. Even though AMF colonization leads to systemic responses throughout the plant, until recently no AMF-specific metabolic response has been detected in plant parts other than in roots (Aliferis et al., 2015; Hill et al., 2018). The described protocol is based on a MeOH extraction of leaf tissue followed by Ultra High Performance Liquid Chromatography Mass Spectrometry (UHPLC-MS) analysis as described by Wang et al. (2018). The concentrations of foliar 11-hydroxy- and 11-carboxyblumenol C derivatives are not detectable in non-mycorrhized plants and are positively and quantitatively correlated with AMF root colonization and are transported from roots to the leaves after the formation of root-AMF associations (Wang et al., 2018). This protocol

facilitates an HTP, non-destructive and quantitative characterization of AMF associations in various model and agricultural crop plant species.

Materials and Reagents

1. Pipette tips
2. 96-well microplates with full skirt (Sapphire, Greiner Bio-One, catalog number: 652270)
3. (Optional) Individual tubes

Note: Individual tubes can be used instead of 96-well BioTubes™ for small batches of samples.

 - a. 2 ml Eppendorf Safe-Lock tubes (Eppendorf, catalog number: 0030120094)
 - b. 1.5 ml screw neck vials N9 (Macherey-Nagel, catalog number: 702282)
 - c. N9 PP screw caps (Macherey-Nagel, catalog number: 702287.1)
 - d. Steel balls Ø 4 mm (ASKUBAL, G100-1.4034, catalog number: 503012)
4. Sealing film for 96-well microplates (Zone-free™, EXCEL Scientific, catalog number: ZAF-PE-50)
5. 96-well PCR Plate (ultraAmp, Sorenson™ BioScience Inc, catalog number: 21970)
6. Domed 8-strip PCR caps (Eppendorf, catalog number: 0030124839)
7. Steel balls Ø 3 mm (ASKUBAL, G100-1.4034, catalog number: 505001)
8. Leaf material
9. Liquid nitrogen
10. MilliQ water
11. Deuterated internal standard: D₆-ABA (HPC Standards GmbH, 10 µg ml⁻¹ in MeOH)
12. Acetonitrile (VWR International, HiPerSolv CHROMANORM® for LC-MS, catalog number: BDH83640.100E)
13. Formic acid (Fluka, for mass spectrometry, catalog number: 94318)
14. Methanol (Merck, Gradient grade for LC LiChrosolv®, catalog number: 1060072500)
15. Roseoside (Wuhan ChemFaces Biochemical Co., Ltd., catalog number: CFN98916)
16. Corchoionoside C (Wuhan ChemFaces Biochemical Co., Ltd., catalog number: CFN99859)
17. Blumenol C glucoside (Wuhan ChemFaces Biochemical Co., Ltd., catalog number: CFN99424)
18. Byzantionoside B (Wuhan ChemFaces Biochemical Co., Ltd., catalog number: CFN99871)
19. Extraction buffer with deuterated internal standard D₆-ABA (see Recipes)

Equipment

1. Stainless steel spatula
2. Stainless steel tweezers
3. 96-well tube racks (BioTube™, Simport® Scientific, catalog number: T101-1 and T100-20)
4. Sealing mats for 96-well tube racks (ArctiSeal™, Arctic White LLC, catalog number: AWSM-2002RB)

5. Cooling containers (Heathrow Scientific, True North®)
6. Centrifuge (Eppendorf, model: 5415 R)
7. Multipipette (Multipette® Xstream, Eppendorf, catalog number: 4986000025)
8. Mortar and pestle (Haldenwanger™, Fisher Scientific)
9. Analytical balance (Sartorius, model: BP121S)
10. 8-channel electronic pipette (Eppendorf, Xplorer®, 50-1,200 µl, catalog number: 4861000163)
11. Tissue homogenizer (Geno/Grinder® 2000, SPEX SamplePrep)
12. Cooled centrifuge equipped with 96-well plate rotor (Eppendorf, model: 5804 R, rotor A-2-DWP)
13. UHPLC triple quadrupole MS instrument [Ultimate 3000 RSLC (Thermo Fisher Scientific); EVO-Q Elite™ (Bruker)]
14. UHPLC column (ZORBAX Eclipse XDB-C18, 50 x 3.0 mm, 1.8 µm, Agilent, catalog number: 981757-302)
15. -80 °C freezer

Software

1. MS Data Review Version 8.2.1 (MS Workstation, Bruker Daltonics)

Procedure

Notes:

- a. In order to test the applicability of the method for the analyzed plant/AMF species, it is advised to perform an initial test screen with root tissue as the abundance of blumenol derivatives in root tissue is higher than in leaves.
- b. Blumenol levels can vary in different shoot tissues (Wang et al., 2018). Harvesting tissue samples from leaves at comparable developmental stages will reduce variation and allow better comparisons between plants.
- c. Blumenol levels reliably indicate AMF colonization 3 weeks after inoculation (Wang et al., 2018).

A. Collection and preparation of leaf material

1. Harvest leaves and immediately freeze in liquid nitrogen using stainless steel tweezers. Store at -80 °C until processing the samples.
2. Grind the frozen leaf material with mortar and pestle under liquid nitrogen.
3. Aliquot approximately 100 mg leaf material with a pre-cooled stainless steel spatula into liquid nitrogen-precooled and pre-weighted 96-well BioTube™ racks containing two steel balls (Ø 3 mm). Record the exact mass and leave the samples on liquid nitrogen for extraction or store at -80 °C.

Note: Instead of 96-well BioTubes™, 2 ml Eppendorf tubes equipped with two steel balls (Ø 4 mm) can be utilized.

B. Extraction

1. Add 800 μ l of ice-cold extraction buffer containing the internal standard D₆-ABA to each tube with an 8-channel pipette. Replace the tube caps with a rubber sealing mat.
Note: Samples should be kept on ice during the extraction procedure.
2. Homogenize the samples in a Geno/Grinder® for 60 s at 1,000 strokes per minute (Geno/Grinder® 2000 setting: 1x at 000).
3. Centrifuge the samples at 2,000 $\times g$ for 20 min at 4 °C, transfer the supernatant to a new 96-well BioTube™ rack or Eppendorf tubes without steel balls and centrifuge again under the same conditions.
4. Transfer 100 μ l of the supernatant into skirted 96-well microplates and close wells with sealing film for LC-MS/MS analysis.
5. As the sealing film is not suitable for long-term freezer storage, transfer 190 μ l of the supernatant into 96-well PCR plates and seal with 8-strip caps as freezer backup.
Note: In case Eppendorf tubes are used, transfer 700 μ l of the supernatant to 1.5 ml screw neck vials (vials are stored in the freezer for re-analysis).
6. Prepare a mixed quality control (QC) sample for each 96-well plate by combining 10 μ l aliquots of each sample of the plate in a 1.5 ml screw neck vial.
7. Use the extraction buffer as blank and for signal background calculations.

C. UHPLC-MS/MS

For the chromatographic separation, utilize an Agilent ZORBAX Eclipse XDB-C18 column. The mobile phase consists of 0.1% (v/v) acetonitrile and 0.05% (v/v) formic acid in MilliQ H₂O for solvent A and 100% methanol as solvent B. The mobile phase gradient of the UHPLC method is shown in Table 1. The UHPLC instrument parameters comprise:

Flow rate	0.5 ml min ⁻¹
Sample tray temperature	10 °C
Sample injection volume	5 μ l
Column temperature	42 °C

Table 1. Mobile phase gradient of the UHPLC run

Time [min]	% mobile phase A	% mobile phase B
0.0-1.0	90	10
1.0-1.2	90-65	10-35
1.2-3.0	65-58	35-42
3.0-3.4	58-0	42-100
3.4-4.4	0	100
4.4-4.5	0-90	100-10
4.5-5.5	90	10

The Bruker EVO-Q Elite™ triple quadrupole MS system is used in multiple reaction monitoring (MRM) mode. The heated electrospray ionization (HESI) source settings consist of:

HESI spray voltage	± 4,500 V
Cone temperature	350 °C
Probe temperature	300 °C
Cone gas flow	35
Nebulizer gas flow	60
Probe gas flow	55

System performance and general ESI parameters can be evaluated by injecting a standard solution of related blumenol glycoside compounds: Roseoside (Wuhan ChemFaces Biochemical Co., Ltd.; catalog number: CFN98916), Corchoionoside C (CFN99859), Blumenol C glucoside (CFN99424), Byzantionoside B (CFN99871). Standards for the 11-hydroxy- and 11-carboxyblumenol C derivatives are not commercially available.

The MRM settings for the detection of specific blumenol derivatives are shown in Table 2 and a recording window of 1 min is set at the expected retention time (R_T). The displayed compound table has been tested and found to be widely applicable. Additional markers can be identified in order to extend the method beyond the current list of plant species that have been investigated (Wang et al., 2018):

Barley	<i>Hordeum vulgare</i>
Barrel clover	<i>Medicago truncatula</i>
Common rice	<i>Oryza sativa</i>
Common wheat	<i>Triticum aestivum</i>
Potato	<i>Solanum tuberosum</i>
Stiff brome	<i>Brachypodium distachyon</i>
Tomato	<i>Solanum lycopersicum</i>
Wild tobacco	<i>Nicotiana attenuata</i>

Table 2. Quantifier and Qualifier m/z fragments used to detect blumenol derivatives in plant leaves

Compound name	R _T [min]	Precursor ion m/z [§]	Quantifier m/z [collision energy]	Qualifiers m/z [collision energy]
11-hydroxyblumenol C-Glc [*]	2.78	(+) 389.2	209.2 [7.5 V]	227.2 [2.5 V], 191.1 [12.5 V], 163.1 [15.0 V], 149.1 [17.5 V]
11-hydroxyblumenol C-Glc-Glc [*]	2.47	(+) 551.3	209.2 [10.0 V]	389.2 [2.5 V], 227.2 [7.5 V], 191.1 [15.0 V], 149.1 [20.0 V]
11-carboxyblumenol C-Glc [*]	3.17	(+) 403.2	195.1 [12.5 V]	241.2 [2.5 V], 223.2 [7.5 V], 177.1 [15.0 V]
	3.17 [‡]	(+) 241.2	195.1 [10.0 V]	223.2 [5.0 V], 177.1 [15.0 V]
11-carboxyblumenol C-Glc-Glc [†]	3.10	(+) 565.2	195.1 [15.0 V]	403.2 [2.5 V], 241.2 [4.5 V], 223.2 [15.0 V]
11-carboxyblumenol C-Mal-Glc [†]	3.60	(+) 489.2	195.1 [12.5 V]	241.2 [2.5 V], 223.2 [7.5 V], 177.1 [15.0 V]
abscisic acid (ABA) ^{**‡}	4.00	(-) 263.2	153.0 [9.0 V]	
blumenol A-Glc ^{**‡}	2.46	(+) 387.2	207.1 [8.0 V]	225.2 [5.0 V], 149.1 [18.0 V], 135.1 [16.0 V], 123.1 [23.0 V]
	2.46	(-) 385.2	153.1 [14.0 V]	
D ₆ -ABA [¶]	4.01	(-) 269.2	159.0 [10.0 V]	

[§] Ionization polarity is indicated in parentheses.

^{*} Verified by NMR.

^{**} Verified with authentic standard.

[‡] The fragmentation of the m/z 241.2 aglycon precursor [M+H-Glc]⁺ allows for a sensitive MRM detection in addition to the MRM of the m/z 403.2 molecular ion [M+H]⁺.

[†] Blumenol A and abscisic acid are not induced by AMF (Wang et al., 2018) and can be used as internal standards to evaluate the overall functionality of the carotenoid biosynthesis in the analyzed plant as well as providing valuable information about instrument performance.

[¶] The identity of 11-carboxyblumenol C-Glc-Glc and 11-carboxyblumenol C-Mal-Glc detected in rice has not been confirmed.

[¶] Internal Standard (typically showing 20-30% relative standard deviation after full extraction/analysis procedure).

The prepared QC samples will be analyzed repeatedly after every 15 to 20 samples with the identical UHPLC-MS/MS method. Comparisons of the QC runs will allow monitoring instrument performance and detecting retention time shifts or changes in mass spectrometer sensitivity in larger sample batches.

Data analysis

EXAMPLES of the blumenol derivative signals detected in leaves of barley (*Hordeum vulgare*) and tomato (*Solanum lycopersicum*) plants with and without AMF colonization are shown in Figure 1.

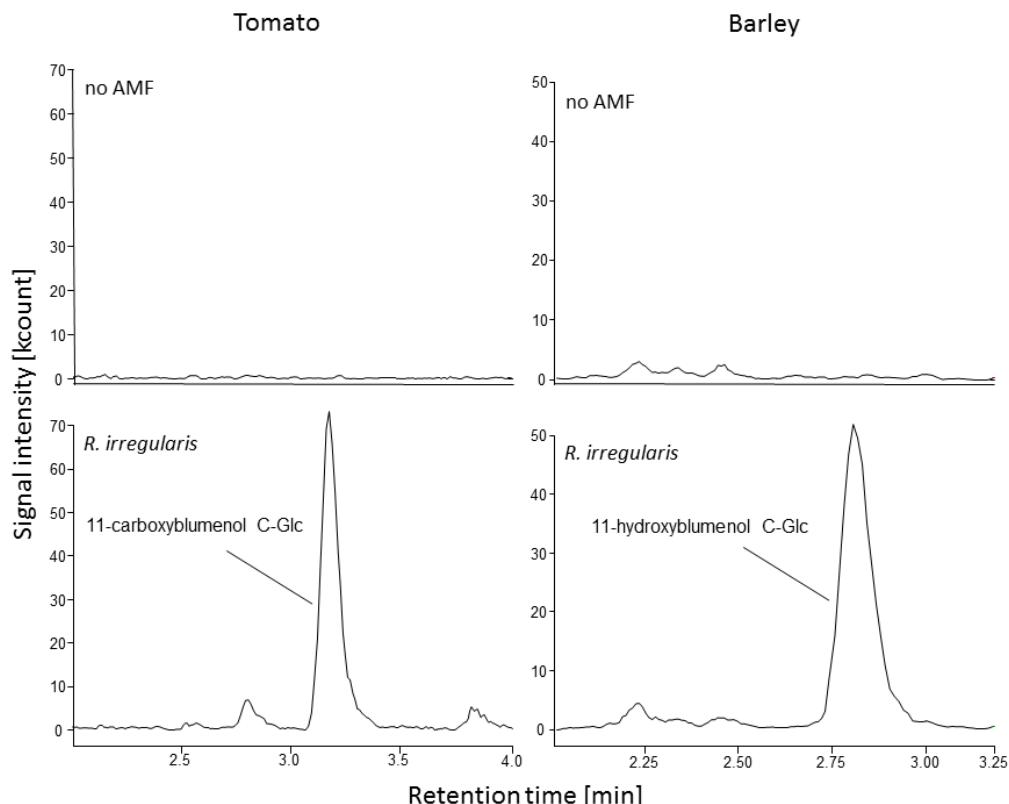


Figure 1. Chromatographic output for blumenol derivatives in different crop plants.

Blumenol derivatives were extracted from leaf tissue of control plants (no AMF) and plants inoculated with *Rhizophagus irregularis*. 11-carboxy- and 11-hydroxyblumenol C-Glc were detected in AMF-colonized tomato (*Solanum lycopersicum*) and barley (*Hordeum vulgare*) plants, respectively. Details of the inoculation procedure can be found in Wang et al. (2018).

Peak area integration for the targeted compounds and the internal standard is carried out via the software MS Data Review Version 8.2.1 (MS Workstation, Bruker Daltonics). The analyte peak area is normalized to the internal standard D₆-ABA and concentrations of blumenol derivatives are calculated as D₆-ABA equivalents (ng mg⁻¹ fresh mass) using the following equation:

$$\frac{\left(\frac{PA_{analyte}}{PA_{D_6-ABA}} \times mass_{D_6-ABA}\right)}{mass_{sample}}$$

$PA_{analyte}$ and PA_{D_6-ABA} represent the peak areas (in counts) of the target analyte and internal

standard, respectively.

$mass_{D_6-ABA}$ is the amount of internal standard (in ng) that is introduced to the sample via the extraction buffer.

$mass_{sample}$ corresponds to the fresh mass (in mg) of the leaf tissue sample.

Recipes

1. Extraction buffer including internal standard D₆-ABA

200 ml MilliQ H₂O

800 ml MeOH (gradient grade for LC)

1.25 ml of 10 ng μ l⁻¹ D₆-ABA (final concentration of 10 ng per 800 μ l extraction buffer)

Acknowledgments

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Competing interests

The procedure has been filed under PCT patent application PCT/EP2019/054738 with the European Patent Office.

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Tensile Testing Assay for the Measurement of Tissue Stiffness in *Arabidopsis* Inflorescence Stem

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[Abstract] Lignocellulosic biomass is a versatile renewable resource for fuels, buildings, crafts, and biomaterials. Strategies of molecularly designing lignocellulose for industrial application has been developed by the discoveries of novel genes after the screenings of various mutants and transformed lines of *Arabidopsis* whose cell walls could be modified in the inflorescence stem, a model woody tissue. The mechanical properties are used as a quantitative index for the chemorehological behavior of the genetically modified cell wall in the tissue. This parameter can be measured with tensile or bending tests of tissue explants, the vibration analysis of tissue behavior or using atomic force microscopy to probe the tissue surface. Here, we describe in detail the procedure to determine the stiffness of methanol-fixed, rehydrated and pronase-treated inflorescence explants with a tensile testing machine based on classical methods for the determination of cell wall extensibility.

Keywords: *Arabidopsis*, Cell wall, Compliance, Inflorescence stem, Mechanical properties, Stiffness, Tensile tests, Young's modulus

[Background] Plant biomechanics can be measured with different methods such as a tensile test (Burgert et al., 2003; Cosgrove, 2011), a bending test (Shah et al., 2017), vibration analysis (Niklas and Moon, 1988, Nakata et al., 2018) and atomic force microscopy (Radotić et al., 2012). These techniques are available to quantify the stiffness and strength properties of materials. Stiffness is defined by the force required to displace (stretch) the material over a unit length, while the strength is regarded as the amount of force required to ultimately rupture the material. The stiffness of a structure is affected by the material properties such as lignocellulosic composition and the bio-structural geometry such as a thickness of cell wall (Shah et al., 2017). The biomechanical tests designed to measure these properties are frequently used in timber grading for commercial sale as well as in tree breeding programs to select and breed superior trees with increased stiffness and strength. Such tests can be also used in *Arabidopsis thaliana* to identify candidate genes with effects on strength and stiffness in mutant screening as a wood model (Strabala and MacMillan, 2013, Figures 1A-1C). In fact, the tensile test was used to estimate the stiffness of inflorescence stems in *Arabidopsis* in our previous study (Sakamoto et al., 2018). The protocol in this article provides the procedure to measure the stiffness of mature regions of *Arabidopsis* inflorescence stem with a tensile testing machine (Figure 1D). It is based on the method

to measure the cell wall extensibility (compliance) of tissue explants of dicot seedling stems and monocot coleoptiles (Olson *et al.*, 1965; Cleland, 1967) performed intensively between the 1960s and the 1980s (Taiz, 1984; Masuda, 1990). This has been optimized to the determination of the stiffness of inflorescence stem in *Arabidopsis* and described in this article.

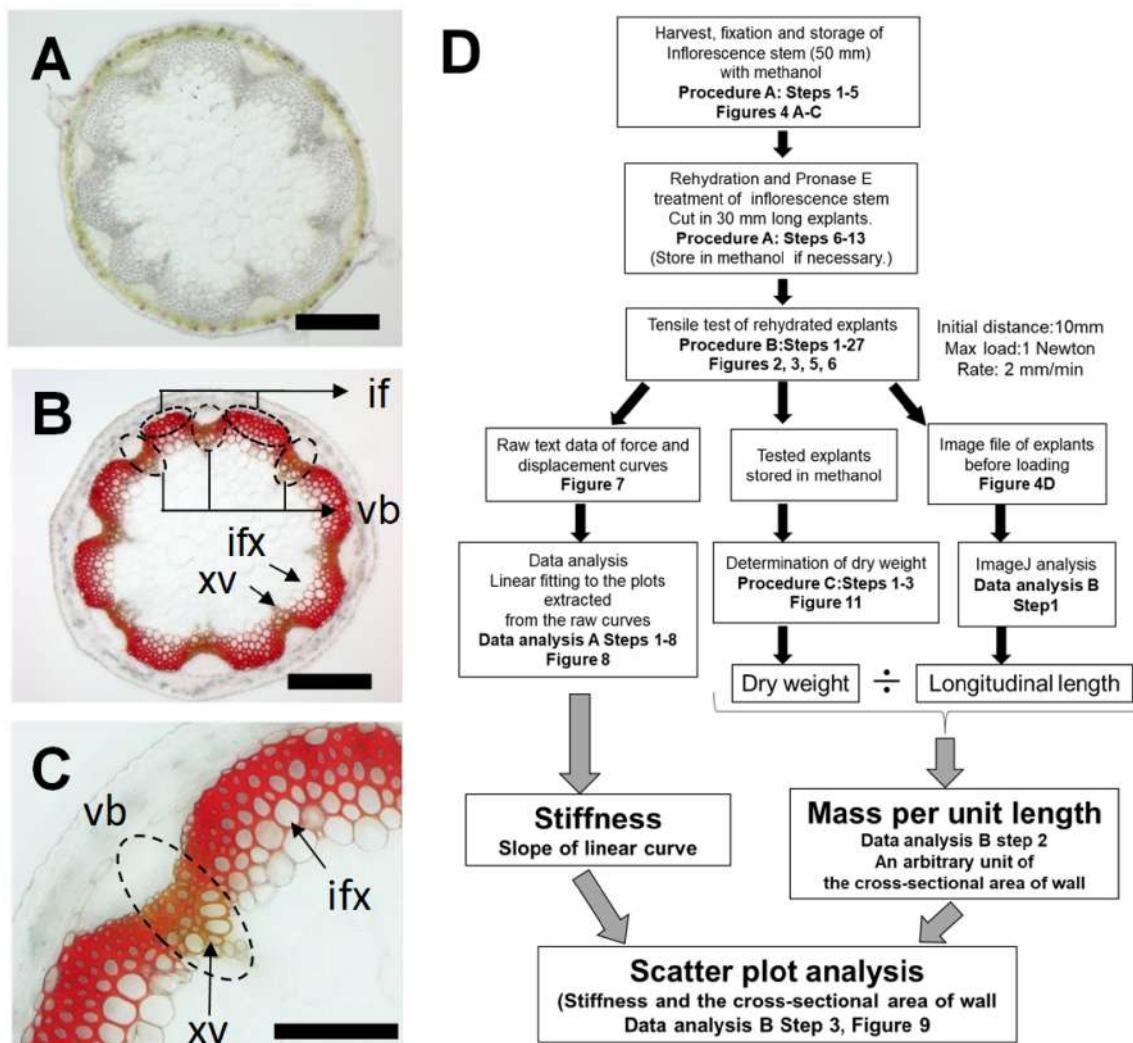


Figure 1. Overview of tensile test to measure the tissue stiffness of a model woody tissue in *Arabidopsis*. A. Cross sections of mature regions of *Arabidopsis* inflorescence stem as a model woody tissue. B and C. Stained cross section with Mäule reagent. Interfascicular fiber (If), Vascular bundles (vb), xylem vessels (xv), interfascicular xylem fibers (ifx). Bars indicate 200 μ m. D. Flow chart of tensile testing assay for the measurement of tissue stiffness in *Arabidopsis* inflorescence stem.

Materials and Reagents

1. Transparent tape
2. PYREX® 55 ml Screw Cap Culture Tubes with PTFE Lined Phenolic Caps, 25 x 150 mm

(Corning Co. Ltd., catalog number: 9826-25)

3. Plastic transfer pipet (Transparent LDPE, 4 ml, Thin stem, Thermo Fisher Scientific Co. Ltd., catalog number: 13459118)
4. Razor blade (Hi-Stainless Single Edge Blade, FEATHER Safety Razor Co. Ltd., catalog number: FHS-10)
5. Plastic Petri dish (90 mm x 14.2 mm) (Thermo Fisher Scientific Co. Ltd., catalog number: 5184E)
6. Kimtowel wipes (Kimberly-Clark Co. Ltd.)
7. Kimwipe paper strips (Kimberly-Clark Co. Ltd., ca. 2 mm x ca.10 mm in length) (Figure 2)
8. 2 ml microtube (Eppendorf Co. Ltd., catalog number: 0030120094)
9. Aluminum foil
10. Inflorescence stem of 8 week-old *Arabidopsis thaliana*
11. Methanol (Kishida Chemical Co. Ltd. Japan, catalog number: 000-48666)
12. KH₂PO₄ (Nakalai Co. Ltd. Japan, catalog number: 09582-75)
13. K₂HPO₄ (Nakalai Co. Ltd. Japan, catalog number: 09583-65)
14. Pronase E (PE) from *Streptomyces griseus* (Product name is "Actinase E". Nacalai Co. Ltd. Japan, catalog number: 29003-51), store at 4 °C
15. SDW: Sterilized ultrapure water (e.g., Milli-Q)
16. Potassium phosphate buffer for PE solution (see Recipes)
17. PE solution (see Recipes)



Figure 2. Kimwipe paper strips used to fix sample with the clamps (ca. 2 mm x 10 mm in length, hand-made)

Equipment

1. Tensile testing machine for max. 500 Newton (N) (T.S.E. Co. Ltd. Yokohama, Japan, Auto Com Series, catalog number: AC-500N-CM, Figure 3A, see Note 1)
2. Load cell for max.10 N (T.S.E. Co. Ltd., Catalog number: TC-10N-B) (Figure 3A)
3. Upper and lower fiber clamps in lever action (Figures 3B and 3C, Clip jaw 5N, A & D Co. Ltd., Tokyo, Japan, catalog number: J-TZM-5N)

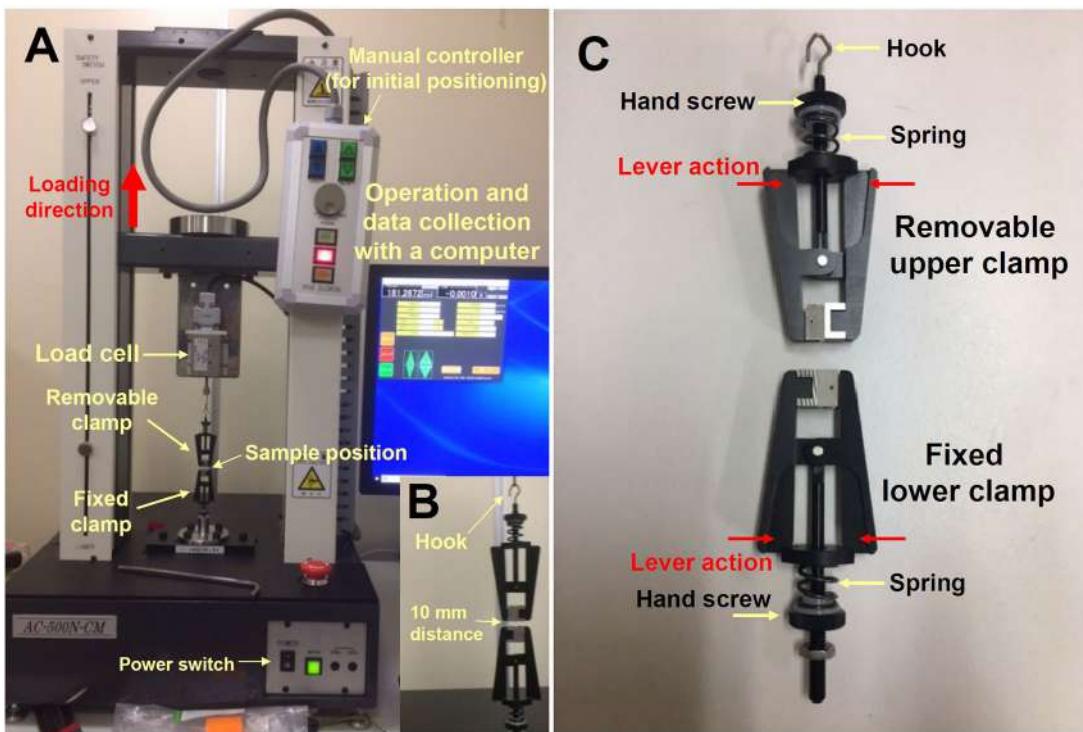


Figure 3. Tensile testing machine and its clamps. A. Tensile testing machine for maximal 500 N force. B. Removable clamp with a hook and fixed clamp. Set the zero point of the load in this position of the removable clamp. C. Top-removable and bottom-fixed fiber clamps. The clamp can be opened by lever action and sample should be fixed with hand screw tightly.

4. Oven (37 °C or 60 °C, EYELA Co. Ltd., Japan, Forced Air Flow Oven, WFO420)
5. Water bath (TR-1a, ASONE Co. Ltd., Japan, catalog number: 1-5832-31)
6. Chemical fume hoods
7. Analytical balance (Mettler Toledo Co. Ltd., Balance XPR26, catalog number: 30355476)
8. Universal Anti-Static Kit U-electrode (Mettler Toledo Co. Ltd., catalog number: 11107767)
9. Sharp-Pointed Dissecting Scissor (Thermo Fisher Scientific. Co. Ltd., catalog number: 08-940)
10. Digital camera
11. Ruler (up to 150 mm)
12. Tweezer (EBL Co. Ltd., model: #202)

Software

1. Operation and analysis software for the tensile testing machine (T.S.E. Co. Ltd., Yokohama, Japan, UTPS-STD Single for windows 10, English version is available)
2. Microsoft Excel (<https://products.office.com/en-au/excel>)

Procedure

A. Plant material preparation (requires 2-3 days after the harvest of tissue)

1. Excise the basal parts (roughly 30 mm from bottom of stem) of inflorescence stems from 8 week-old *Arabidopsis* with scissors (Figures 4A-4C) and immediately and completely submerged the whole part of explant in 40 ml methanol in PYREX® 55 ml screw cap culture tubes. Plants are grown under 16-h light ($60-80 \mu\text{mol m}^{-2} \text{s}^{-1}$)/8-h dark cycle in a constant 23 °C plant room with no humidity control.

Note: More than fifteen explants of inflorescence stem are necessary for the evaluation of one plant line (see Note 2).



Figure 4. Stem segment taken from basal part of the inflorescence stem of *Arabidopsis*.

A. Eight-weeks old plant grown in soil. The basal end was cut at the position of red arrowhead with a pair of scissors. B. Harvested and fresh inflorescence stem. Excised stem segment which should be cut at the position of arrowhead. All branches should be removed. C. Digested fresh segment of inflorescence stem which should be submerged into methanol immediately. D. 30 mm-length inflorescence-stem segment after boiled in methanol, rehydration and Pronase E. Treatment. Longitudinal and vertical lengths of each sample should be determined later by Image J.

2. Close the cap of culture tube tightly to avoid a methanol explosion from the tube during the heat treatment described in Step A3.
3. Put the Pyrex glass tubes containing explants dipped in methanol into the water bath at 80 °C in the chemical fume hood. Incubate them for 15 min (see Note 3).
4. After cooling the tubes at room temperature, discard the green-colored methanol extract and add 40 ml of fresh methanol to the glass tube.
5. Repeat Steps A3 and A4 until explants become white at least more than five times.
6. Wash the methanol-fixed explants with SDW more than five times to remove methanol.

Note: Make sure all explants sink to the bottom in SDW. If some explants are floating in the tube, stand for 5 min and change SDW again.

7. Discard SDW completely in the tube with a plastic transfer pipet.
Note: Don't touch the tissue explants by a transfer pipet to avoid destruction of tissues.
8. Add 40 ml of the Pronase E (PE) solution (see Recipes), which is pre-incubated for 2 h at 37 °C before use, to a glass tube.
Note: Pre-incubation is essential to deactivate the glycosyl hydrolase contaminants in the PE products. Make sure all explants sink to the bottom of 50 ml glass tube in the PE solution.
9. Incubate the glass tube containing explants in PE solution with tightly capped lids in the oven at 37 °C for 18 h (see Note 4).
10. Discard PE solution from the tube.
11. Wash the PE-treated explants with SDW more than three times and remove the remaining solution from the 50 ml-glass tube as much as possible with a plastic transfer pipet.
Note: Don't touch the tissue explants with a transfer pipet to devoid any destruction of tissues.
12. Trim the basal end of explants with a razor blade to make them exactly 30 mm in length (Figure 4D).
13. Determine the force and displacement curve of inflorescence explant (Procedure B) or store the 30 mm-length tissue explants in methanol at room temperature until use. Wash the explants with SDW at least three times before the experiment if the explants are stored in methanol (Step 10 of Procedure B)

B. Determination of the force and displacement curve of inflorescence explants (ca. 5 min for the measurement of one sample).

1. Turn on the tensile tester and the computer (Figure 3A)
2. Set the “tensile test mode” in the operation software and the data sampling method to X-T mode with the maximal resolution of distance (0.001 mm) according to the manufacturer's manual.
3. Set the initial distance between upper (top) and lower (bottom) fiber clamps to 10 mm after setting the upper clamp (without sample) to the original position (Figures 3B and 6F).
4. Calibrate the load cell (at both 0% and 50% of 10 N) without removing the upper removable clamp according to the manufacturer's manual (Figure 3B).
5. Set the speed of upper movable clamp to 2 mm/min.
6. Set the maximum load to 1 N (ca. 100 g) which is the load when the test is finished.
7. Set the program to return upper movable clamp to the initial position after the test.
8. Designate the data file format and enter the plant-line name of samples and the test number.
9. Prepare 2 ml-micro tubes containing 2 ml fresh methanol in tube rack. Protect the tube label (plant line name and test number) from methanol with transparent tape.
10. If samples have been stored in methanol prior to the test, wash and rehydrate the 30 mm length explants by at least five changes of SDW. Make sure all tissue explants sink to the bottom of the glass tube after the rehydration.
11. Transfer tissue explants into the plastic Petri dish containing 40 ml SDW from the glass tube and keep them in the SDW until needed for the test.

12. Take a tissue explant out from the SDW and immediately take a photograph of the tissue explant with a ruler and the label of the test number and sample name with a digital camera (Figure 4D). Determine the longitudinal and vertical lengths of explants later.

Note: Avoid drying the sample from Steps B12 to B24.

13. Put the tissue explant on Kimtowel wiper to remove excess water on the surface of the explant.
14. Put the 2 mm x ca.10 mm strips of Kimwipe on both ends of the tissue explant (Figure 5A). Fold each paper strip at the middle to hold the both edges of the tissue explant (Figure 5B).

Note: These paper strips are necessary to prevent tissue slippage in the fiber clamps during the test.

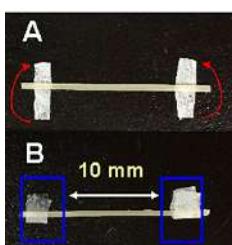


Figure 5. Two small paper strips of Kimwipe are used to prevent the sample slippage in the clamps. A. After putting two paper strips under the sample, fold two paper fragments in the middle to the direction of red arrows. B. After that, fix the parts of the sample, which are indicated by blue boxes, with the upper and lower clamps.

15. Fix one side of the paper strip-covered parts of the explant (Figure 5B) with the removable clamp by the lever action. Make sure the longitudinal axis of the tissue is vertically positioned at the center of the upper clamp (Figures 6A and 6B).
16. Tighten the hand screw of the upper clamp to fix the tissue more. Do not tighten the screw completely to avoid destruction of the tissue (Figure 3C). Make sure the top-removable clamp fitting to the sample without any gap (upper arrowhead in Figure 6C) and any distortion (lower arrowhead and white dashed lines in Figure 6C). The gap and distortion could cause slightly wavy fluctuation of force extension curve during the tensile test. If gap and distortion are found, adjust the clamps and the sample by pushing the attachments of clamp with your thumbs (Figures 6D and 6E). This adjustment is also necessary to fix the sample with the bottom-fixed clamp in Steps B18 and B19.

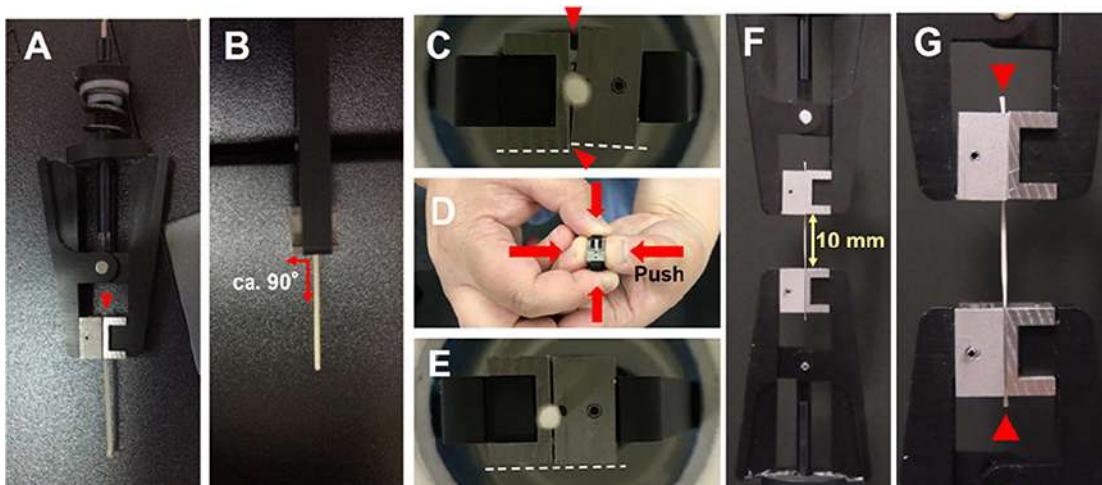


Figure 6. Tissue sample fastened between two clamps. A. Fix the sample with the upper removable clamp first (front view). See the position of one end of sample (Arrowhead). B. Make sure the angle between the sample and the edge of the upper clamp is ca. 90 degree (Side view). C-E. Make sure the top-removable and bottom-fixed clamps fitting to the sample without any gap (indicated by the upper arrowhead in C) and any distortion (indicated by the lower arrowhead and white dashed line in C). F and G. After hanging the upper clamp with the sample on the hook of the load cell, fix the sample with the lower clamp. Make sure both upper and lower ends of the sample protruding from clamps a little (Arrowheads in G).

17. Hang the upper clamp with the explant on the hook of the loading cell.
18. Fix another side of the paper strip-covered part of the explant with the lower clamp (Figure 6F). Make sure the longitudinal axis of the tissue is vertically positioned at the center of the lower clamp as for the upper clamp. Both ends of the sample should extrude a little from the clamps (Arrowheads in Figure 6G).
19. Tighten the hand screw of the lower clamp to fix the tissue securely (Figure 3C). Do not tighten the screw completely to avoid destruction of the tissue (see Note 5).
20. Make sure the load between two clamps is almost 0 N (From -0.042 to 0.0183 N, Mean and standard deviation: $-0.0059 \text{ N} \pm 0.0092$ in our experiments) in order to avoid prestretching before the test. A strong prestretch could cause the plastic deformation of the sample before the estimation of stiffness (Cleland, 1967).
21. If the load value before the test is not ignorable, loosen the hand screw of the lower clamp slightly. Repeat Steps B18 and B19 to get the load value close to 0. Alternatively, change the distance between two clamps to shorter than 10 mm with a manual controller (Figure 1A) to let the load value close to zero without touching the lower clamp (-0.01 mm to 0.05 mm in our experiments). In the latter case, record the changed distance between two clamps.
22. Start loading the sample to 1 N by lifting the upper clamp.
23. After reaching to 1 N, the loading should be stopped mechanically and the upper clamp should return to the initial position (10 mm distance) immediately. Prepare the next sample during this

process (from Step B12).

24. After the return of the upper and movable clamp to its original position, remove the tested sample from the upper and lower clamps by loosening the hand screws and levering action.
25. Store each tested sample in methanol in a numbered 2 ml-micro tube.
26. Repeat these steps (from Step B12 to Step B25) for all samples in one plant line.
27. Store the series of all data for one plant line and export the relationship between displacement (mm) and load (N) for all samples in text format. The text data files should be analyzed with Microsoft Excel software or equivalent software (see Data analysis).
28. Turn off the tensile testing machine and the computer.

C. Determination of the sample dry weight

1. Discard methanol from the 2 ml-microtubes containing a tissue explant (stored in Step B24) with a plastic transfer pipet avoiding destruction of the sample.
2. Place the 2 ml-microtubes in the oven at 60 °C for 2 days without capping but with a small piece of aluminum foil sheet to prevent dust contamination.
3. Weigh the dried tested sample with an analytical balance equipped with an anti-static apparatus and the custom-made wind-proof cover (Note 6).

Data analysis

A. Determination of inflorescence stiffness

1. Open the text data file generated at the “Step 27” of Procedure B with Microsoft Excel or equivalent software. (An example of the text data file in our experiments is provided as a [supplemental text files “Col0”](#).)
2. Highlight all cells containing data. In our data, the first column (A) contains values of displacement (mm), and the second column (B) contains values of force (N) in the single tensile test and draw scatter plot using the software function.
3. Observe the force and displacement curves (Figure 7). The curve should have two phases. At first, the displacement is accompanied by little increase of the load. This is, at least in part, due to some slack existing in the explant when it was fixed between the clamps and thus should be removed. Beyond a certain point, load increases in a linear manner as displacement proceeds (Cleland, 1967). Use the force and displacement values in the linear range to calculate stiffness.
4. Draw the curves for all tests and compare the force ranges in a linear manner to extract the representative linear range. For example, we estimated that the representative linear force range was between 0.6 to 0.8 N in our experiment (Figure 7).

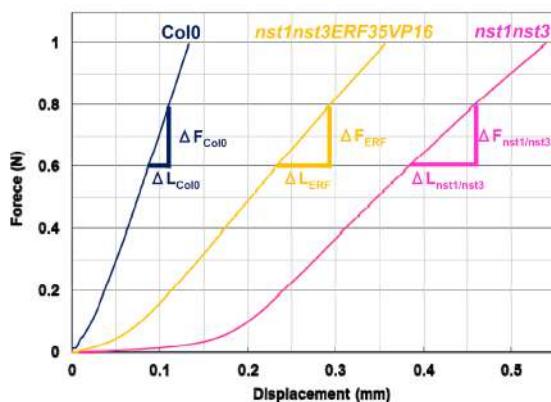


Figure 7. Force and displacement curves for inflorescence explants in different plant lines of *Arabidopsis*. Col0: wild type, *nst1nst3*: double knockout mutant of *NST1* and *NST3* genes (Mitsuda et al., 2007), *nst1nst3ERF35VP16*: *NST3* promoter-driven *ERF035* gene with VP16 activation domain in the double knockout mutant (Sakamoto et al., 2018).

5. Highlight the cells of displacement column and force column in the fixed force range (for example, from 0.6 to 0.8 N) and create a scatter plot.
6. Add the linear fit (a straight line fit) using the software function. In Microsoft Excel, click once anywhere inside the graph area and select the “Layout” tab from “Chart Tools”. Click “Trendline” icon and select the “Linear Trendline” option.
7. To show the equation, click “Trendline” and select “More Trendline Options...” Then check the “Display Equation on chart” and “Display R-squared value on chart” boxes. (Figure 8).
8. The slope of the equation indicates stiffness (N/mm, Note 7). Examine the fitness of a linear plot with R-squared value (R^2 : 0.9969 to 1 in our experiment).

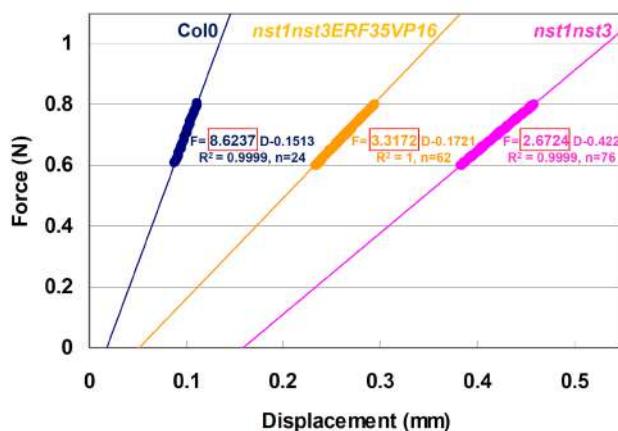


Figure 8. Linear plots and equations of force (F) and displacement (D) curves prepared by Microsoft Excel. Round-shaped actual plots indicate the values between 0.6 and 0.8 N, which were used for the linear fit. “n” indicates the number of used plots. The slope in the equation surrounded by the red box gives the stiffness of each sample. R square-values are also indicated.

- B. Determination of mass per unit length of tissue explants and the estimation of the effects of introduced or mutated genes on the inflorescence-stem stiffness
1. Determine the longitudinal and the vertical length of tissue explants from the photograph taken at Step 12 of Procedure B (Figure 4D).
 2. Divide the dry weight (obtained at the Step 3 of Procedure C) by the longitudinal length of each tissue explant to obtain mass per unit length.
 3. Plot the mass per unit length and the stiffness of the investigated plant lines on a graph to see if there is any trend (*i.e.*, effects of genes on the material properties such as cell wall compositions and/or the bio-structural geometry such as wall thickness, Figure 9).

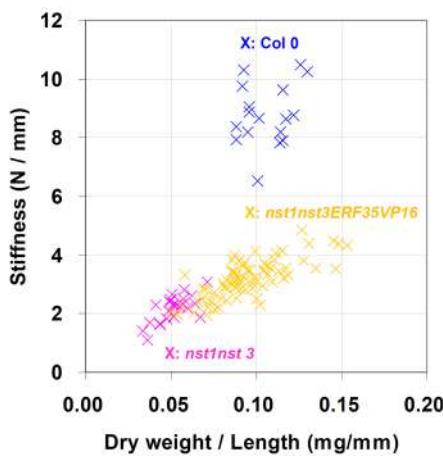


Figure 9. Scatter plot between dry weight/length and force/extension (stiffness) of different genotype plants. Wild-type ($n = 17$ biologically independent samples), *nst1nst3* ($n = 27$ biologically independent samples) and *nst1nst3 NST3pro::ERF035-VP16* ($n = 76$ biologically independent samples) (Sakamoto *et al.*, 2018).

C. Representative data

One set of raw text data of the force and displacement curve of wild-type *Arabidopsis* inflorescence-stem explant is provided as a [supplemental text file](#). Three test results of our experiments (Figures 7 and 8) are also provided as a [supplemental Excel file \(Force and displacement.xls\)](#). For the plot between mass per length and stiffness, see Figure 9.

Notes

1. The tensile testing machine, AC-500N-CM, was exported from Japan to destinations such as Singapore and Thailand before. English manual is available for the operation of this machine with a computer. Other tensile testing machines are also available in the local manufacturer in your country such as Instron (<https://www.instron.us/en-us>). The detailed performance of the tensile testing machine in our laboratory is shown in Table 1.

Table 1. Performance of the tensile testing machine AC-500N-CM

Loading system	Closed loop digital servo mechanism by computer (Windows 10)
Loading capacity:	Maximum: 500 N (50 kgf)
Effective test width:	200 mm
Crosshead stroke:	490 mm
Effective stroke:	250 mm
Crosshead-performance	
Operation:	Auto and manual operations are available.
Speed range:	0.01-500 mm/min
Accuracy	±0.2% of indicated value
Displacement resolution:	5-digit display, ±00.000 mm to ±99.999 mm (X-T-mode)
Return-speed:	0.1-500 mm/min
Load-performance	
Load cell:	10 N (1 kgf) as maximum force level
Load range:	7 steps (1, 2, 5, 10, 20, 50, and 100%) or full auto range
Accuracy:	±1% of indicated value within 1-100% of load range
Load resolution:	5-digit display, 0.001-10.000 N
Load calibration:	Auto-zero and auto-spanning functions
Overload protection	Yes, Auto-stop function works at 102% of full scale.
Sample break detection	Yes
Stroke Limiter	2 points at upper or lower position
Dimensions	390 (W) x 410 (D) x 720 (H) mm

2. We used seventeen plants in the wild type, twenty-seven plants in *nst1nst3* mutant line, fifteen to twenty-two plants for each of four independent lines of transgenic *NST3* promoter-driven *ERF035-VP16* (120 plants in total) in the previous publication (Sakamoto et al., 2018).
3. The heat treatment with methanol kills the inflorescence stem and deactivates endogenous cell wall hydrolases. However, expansin, a wall loosening protein remains active in this treatment (Cosgrove, 2011). There is a method to remove protoplasts from plant tissues without using methanol if it is required to see *in-vitro* effects of exogenously applied expansin or glycosyl hydrolase on the mechanical properties (Cosgrove, 2011). Non-metal materials for the fiber clamps in the tensile testing machine were recommended in some experiments (Cosgrove, 2011). If unexpected results of the mechanical properties are obtained, fixation method of tissues and the appropriate material for the clamps may need to be considered.
4. Pronase E (Actinase E) is a nonspecific protease (Nomoto et al., 1960) and generally used for the removal of proteins from the polysaccharide samples (Schmidt et al., 2014; Higashi et al., 2015). This treatment removes the remaining cytoplasmic proteins and cell wall proteins including expansins from the explants. According to Cleland (1967), pronase treatment removed 85-90% of total protein from the stem explants, which was measured by either Kjeldahl nitrogen or proline, but it was less effective to solubilize the structural wall protein such as extensin; only 45% of the hydroxyproline was removed.

5. If the tightening by the hand screw is not appropriate (too strong or too weak), a slippage or breakdown of the sample could be observed during the loading of the sample (Figure 10).

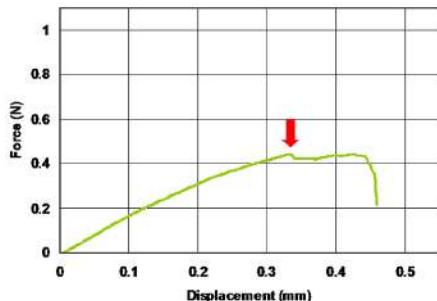


Figure 10. Example of a sample slippage or breakdown. Arrow indicates the displacement where the slippage or the breakdown of the sample has occurred.

6. Dry weight of inflorescence explants (ca. 30 mm in longitudinal length) ranged from 1.02 to 4.91 mg in our experiments. Therefore, we need an analytical balance equipped with an anti-static apparatus and a custom-made wind-proof cover to achieve the stable measurement of the dry weight for each sample (Figure 11).



Figure 11. Analytical balance with custom-made wind-proof cover and antistatic apparatus

7. The obtained equation is also available to calculate the cell wall compliances of tissues by using the mass per unit length as an arbitrary unit of the cross-sectional area (Cleland, 1967). The cell wall compliance is a reciprocal of Young's modulus. If each sample is loaded twice to obtain first and second force extension curves, compliances of elastic extensibility and plastic extensibility can be determined from these two curves (Masuda, 1969; Cleland, 1984; Park and Cosgrove, 2012; Phyo *et al.*, 2017).

Recipes

1. Potassium phosphate buffer for PE
 - a. Dissolve 14.3 g KH₂PO₄ and 25.5 g K₂HPO₄ into SDW and adjust the entire volume to 500 ml to obtain 0.5 M potassium phosphate buffer (pH 7.0)
 - b. Autoclave the buffer stock at 121 °C for 15 min
2. PE solution
 - a. PE powder bottle must be at room temperature before use to avoid its moisture absorption
 - b. Dissolve 0.02 g of PE powder in 85 ml of SDW
 - c. Add 5 ml of ethanol and 10 ml 0.5 M potassium phosphate buffer stock
 - d. The prepared PE solution should be incubated for 2 h at 37 °C to deactivate possibly contaminating glycosyl hydrolases
 - e. After incubation, use PE solution for the protein removal from tissue explants

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Competing interests

The authors declare no competing interests.

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Non-aqueous Fractionation (NAF) for Metabolite Analysis in Subcellular Compartments of *Arabidopsis* Leaf Tissues

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[Abstract] The accurate determination of metabolite distribution in subcellular compartments is still challenging in plant science. Various methodologies, such as fluorescence resonance energy transfer-based technology, nuclear magnetic resonance spectroscopy and protoplast fractionation allow the study of metabolite compartmentation. However, large changes in metabolite levels occur during such procedures. Therefore, the non-aqueous fractionation (NAF) technique is currently the best method for the study of *in-vivo* metabolite distribution. Our protocol presents a detailed workflow including the NAF procedure and quantification of compartment-specific markers for three subcellular compartments: ADP glucose pyrophosphorylase (AGPase) as plastidic marker, phosphoenolpyruvate carboxylase (PEPC) as cytosolic marker, and nitrate and acid invertase as vacuolar markers.

Keywords: *In-vivo* metabolite distribution, Non-aqueous fractionation (NAF), Compartment-specific markers

[Background] Metabolic activity and functionality require precise compartmentation of metabolism in eukaryotic cells. Despite knowing the spatial organization of metabolism within the plant cells, deciphering localization of the metabolites is still challenging due to metabolite redundancy, leakage, rapid turnover, futile cycling, and sophisticated transport systems and storage (Lunn, 2007; Klie et al., 2011; Sweetlove and Fernie, 2013). Several methods have been established to investigate metabolite compartmentation, among them non-aqueous fractionation (NAF) has been widely used for decades to determine the *in-vivo* subcellular metabolite distributions in eukaryotic cells. Firstly developed in animal sciences (Elbers et al., 1974), NAF was subsequently applied to plant leaf material from spinach (Gerhardt and Heldt, 1984 and 1987), bean (Sharkey and Vanderveer, 1989), maize (Weiner and Heldt, 1992) or barley (Winter et al., 1993). NAF is also successfully applied to rose petals (Yamada et al., 2009) and potato tubers (Farre et al., 2001). More recently, NAF has also been optimized to study metabolite compartmentation in the model plant *Arabidopsis thaliana* (Fettke et al., 2005; Krueger et al., 2009 and 2011; Klie et al., 2011; Arrivault et al., 2014; Shapiguzov et al., 2019). The NAF procedure prevents modification of metabolite levels (for example due to enzymatic activity) by having all steps performed at extremely low temperatures or under anhydrous conditions (Gerhardt and Heldt, 1984). After quenching plant material in liquid nitrogen, the frozen material is dehydrated by freeze drying, homogenized, and then applied to a gradient made of anhydrous organic solvents prior to centrifugation and subsequent collection of fractions. Subcellular compartments are separated on the basis of density in the centrifugation step and the fractions collected are enriched in organelles (such as chloroplasts,

cytosol or vacuole; Gerhardt and Heldt, 1984; Lunn, 2006). By correlating the compartment-specific marker distributions with metabolite distributions over the gradient, it is possible to calculate compartment-specific metabolite distributions (Gerhardt and Heldt, 1984; Klie *et al.*, 2011; Krueger *et al.*, 2011). The software tool BestFit enables such calculations (Krueger *et al.*, 2011 and 2014; Klie *et al.*, 2011). Recently, a benchtop NAF method has been established and is adapted for a relatively small amount of tissue material (Fürtner *et al.*, 2016). This benchtop NAF method can be used as an alternative to the conventional NAF procedure. However, we still believe that the conventional NAF procedure is the method of choice for the determination of *in vivo* metabolite distribution among cellular compartments as (i) the plastidic and vacuolar compartments are more clearly separated on the density gradient used, and (ii) the higher amount of starting material leads to more material in each obtained fraction, giving the advantage to increase the number of sub-aliquots per fraction (thus increasing the range of measurements which can be done) and also to apply less sensitive quantification methods than mass spectrometry-based methods. A good example of the use of the conventional NAF procedure is a study performed by Arrivault *et al.* (2014) in which the distribution of about 1,000 proteins and 70 metabolites, including 22 phosphorylated intermediates in *Arabidopsis thaliana* rosette leaves were analyzed, using the conventional NAF combined with MS-based approaches.

The protocol presented here is based on the method described by Krueger *et al.* (2014) with minor modifications. In our method, we provide a complete workflow for the NAF procedure in *Arabidopsis thaliana* and detailed information for the analysis of compartment-specific marker enzymes and metabolites which can be assigned to three subcellular compartments: ADP glucose pyrophosphorylase (AGPase) as plastidic marker, phosphoenolpyruvate carboxylase (PEPC) as cytosolic marker and nitrate and acid invertase as vacuolar markers.

Materials and Reagents

A. NAF

1. Plastic lids (Zinsser Analytic, catalog number: 3071400)
 2. 20 ml Polyvial—one per sample (Zinsser Analytic, catalog number: 3071400)
 3. Steel balls:
 - 3 mm (Askubal, catalog number: 3 mm G100 1.4034)
 - 5 mm (Askubal, catalog number: 5 mm G100 1.4034)
 - 7 mm (Altmann, catalog number: KU.7G80-1.3541)
- Note: We recommend using steel balls of three different diameters 3, 5, and 7 mm (two of each size in each 20 ml Polyvial).*
4. Steel caps (custom made, please check Figure 1)
 5. 50 ml Falcon tubes—10 per sample (SARSTEDT, catalog number: 62.554.502)
 6. Paper tissue (KimWipes)
 7. pH-indicator paper (Merck, catalog number: 1.10962)
 8. Polyallomer centrifuge tubes (Beckman Coulter, catalog number: 326823)

9. Nylon mesh filter 20 µm pore size (SEFAR Nitex®, catalog number: 03-20/14)
10. 1.5 ml screw cap tubes—11 per sample (SARSTEDT, catalog number: 72.692)
11. Activated molecular sieve 4 Å (Carl Roth, catalog number: 1318-02-1)
12. Plastic Pasteur pipette (NeoLab, catalog number: 2600111)
13. Glass pipettes (10 ml, 20 ml)
14. Glass funnel
15. Metal rack
16. *Arabidopsis thaliana* leaf material
17. Liquid nitrogen
18. n-Heptane (Carl Roth, catalog number: 142-82-5)
19. Tetrachloroethylene (Carl Roth, catalog number: 127-18-4)
20. Liquid nitrogen (~5 L per sample)

Note: the required liquid nitrogen for harvesting (1 L), grinding (2 L), and aliquoting (2 L) samples suggested here is a rough estimate, since the amount of nitrogen needed will depend on several factors. Please consider that while planning your experiments.

B. Measurement of metabolite markers

1. 96 Deep-well plate (Sarstedt, catalog number: 82.1970.002)
2. Steel balls (5 mm)
3. Tubes holder for the Retsch mill (Retsch, catalog number: 22.008.0008)
4. Ethanol (VMR Chemicals, catalog number: 20821.330)
5. HEPES (CAS: 7365-45-9)
6. NaOH (Carl Roth, catalog number: 6771.1)
7. Potassium phosphate buffer 1 M, pH 7.5
 - a. K₂HPO₄ (Sigma-Aldrich, CAS: 7758-11-4)
 - b. KH₂PO₄ (Sigma-Aldrich, CAS: 7778-77-0)
8. NADPH (Roche, CAS: 2646-71-1)
9. Nitrate reductase (Roche, catalog number: 10981249001)
10. Phenazine ethosulfate (PES) (Sigma-Aldrich, CAS: 10510-77-7)
11. Sulfanilamide (Sigma-Aldrich, CAS: 63-74-1)
12. N-(1-Naphthyl)ethylenediamine dihydrochloride (NNEDA) (Sigma-Aldrich, CAS: 1465-25-4)
13. KNO₃ (Sigma-Aldrich, CAS: 7757-79-1)
14. Nitrate Standards (see Recipes)
0, 0.2, 0.4, 0.8, and 1.6 mM in 70% ETOH with 10 mM MES pH 5.9

C. Measurement of enzyme markers

For the full description and order details of the reagents used in the measurements of enzyme activities, please see [Supplemental Table 1](#).

1. PVPP

2. Deionized water
3. MgCl₂
4. EDTA
5. EGTA
6. Benzamidine
7. ε-aminocapronic acid
8. BSA (protease free)
9. Leupeptin
10. DTT
11. PMSF
12. Triton X-100
13. Glycerol
14. NaF
15. 3-phosphoglycerate
16. AGPase assay mix
17. PPi
18. Glycerokinase
19. ADP-glucose
20. GPOX
21. GDH
22. NaHCO₃
23. PEP
24. Malate dehydrogenase
25. MTT
26. Alcohol Dehydrogenase
27. Acid invertase assay Buffer
28. Glucose oxidase
29. Horse Radish Peroxidase
30. Amplex Red
31. DMSO
32. Nitrate standards (see Recipes)
33. Extraction buffers (see Recipes)
 - a. Extraction Buffer 10x
 - b. Extraction Buffer 1x
34. AGPase activity (see Recipes)
 - a. AGPase assay buffer
 - b. AGPase assay mix
 - c. AGPase determination mix
35. PEPC activity (see Recipes)

- a. PEPC assay Buffer
 - b. PEPC assay Mix
 - c. PEPC determination mix
36. Acid Invertase activity (see Recipes)
- a. Acid invertase assay Buffer
 - b. Acid invertase Assay Mix
 - c. Acid invertase Determination mix

Equipment

A. NAF

1. Capper/decapper machine (custom made, see Figure 1)
2. Retsch mill (Retsch®, model: MM400)
3. Freeze dryer (Christ, model: Alpha 2-4)
4. Sonicator (BANDELIN SONOPULS HD 2070)
5. Peristaltic gradient pump (Bio-Rad Econo Gradient Pump, catalog number: 731-9002)
6. Gradient mixer (Bio-Rad Gradient mixer, catalog number: 731-8323)
7. Centrifuge (Beckman Coulter, model: Allegra X-15R)
8. Rotor (Beckman Coulter, model: SX4750A)
9. Ultracentrifuge (Beckman Coulter, model: Optima™ L80 XP)
10. Swinging bucket rotor (Beckman Coulter, model: SW32Ti)
11. Desiccator
12. Vacuum pump

B. Measurement of metabolite markers

1. Thermomixer (Eppendorf, model: Compact 5350)
2. Centrifuge (Eppendorf, model: 5430)
3. Microplate Spectrophotometer (BioTeK®, Epoch 2)

C. Measurement of enzyme markers

1. Microplate Spectrophotometer (BioTeK®, ELx808)
2. Microplate Fluorescence reader (BioTeK®, Synergy HT)

Software

1. BestFit (Klie et al., 2011; <http://www.csbdb.de/csbdb/bestfit/bestfit.html>)

Procedure

A. Harvesting, grinding and preparation of leaf material

1. Harvest *Arabidopsis thaliana* leaf material and place into a 20 ml Polyvial (containing steel balls of three different diameters 3, 5, and 7 mm, two of each size in each 20 ml Polyvial) pre-cooled in liquid N₂ and snap-freeze in liquid N₂.

Note: If the metabolites of interest have extremely fast turn-over time, plant material should be harvested by cutting rosettes and quenching them instantaneously in a bath of liquid N₂ under the prevailing irradiance. The quenched material is then transferred into a 20 ml Polyvial (containing steel balls) pre-cooled in liquid N₂. For an optimal grinding process, fill the vial to a maximum of 2/3 of its volume with the plant material. For NAF, 4 g of material is necessary, but it is recommended to harvest slightly more in order to have extra material to perform additional analysis in this material (such as quantification of metabolites, see section Data analysis–4).

2. For homogenization, replace the plastic lids of the Polyvials by steel caps and place the Polyvials containing the leaf material into a pre-cooled steel grinding adapter for the Retsch mill (Figures 1A-1D).

Note: Our grinding adapters and steel caps are custom-made for the 20 ml Polyvials. Alternatively, the grinding of the leaf material can be performed by using a pre-cooled mortar and pestle. Make sure that the leaf material is kept frozen during the whole procedure.

3. Homogenize the tissue for 1 min at 25 Hz (two times) in the Retsch mill (Figures 1E and 1F).
Note: Make sure that the ground tissue is very well homogenized (thin powder). We suggest grinding the leaf material twice for 1 min each at 25 Hz. Cool down the vials and adapters in between with liquid N₂ to avoid thawing of the material.

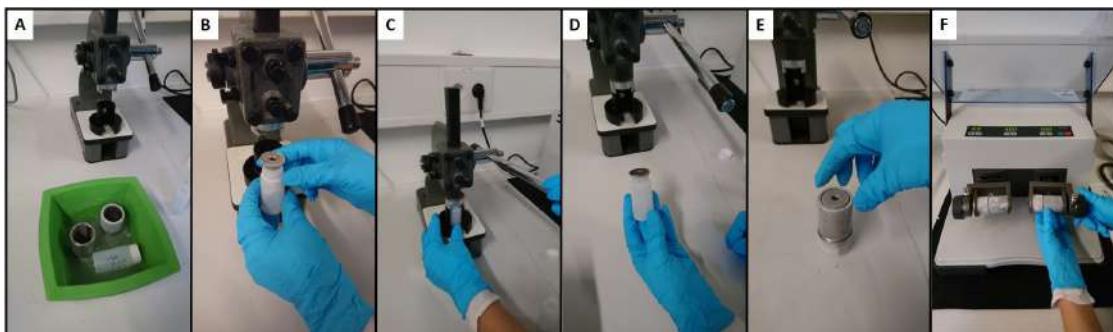


Figure 1. Grinding procedure. A. Cool down the Polyvials, steel grinding adapter, and steel caps in liquid nitrogen. B. Remove the plastic lid of the Polyvials and place the steel cap frozen. C. Use a capper/decapper machine (custom-made) to close the vial with the steel cap. D. Closed vial. E. Place the Polyvial inside steel grinding adapter. F. Place the grinding adapter in the Retsch mill and proceed as in Step A3. After grinding use the same capper/decapper machine for replacing the steel caps for the plastic lids. The samples can be stored at -80 °C or proceed to Step A4. Note that to avoid overheating of the vial, steel caps and grinding adapter deep them in liquid nitrogen or make use of dried ice while preparing a second sample.

4. Weigh ~4 g of frozen tissue into a 50 ml Falcon tube pre-cooled in liquid N₂. The samples can be stored at -80 °C for up to three months. Prepare as many Falcon tubes as necessary (each one will be used for one NAF).

Note: Label the Falcon tubes and their corresponding lids. Record the weights of the empty Falcon tubes (with lids) and the precise amount of plant material weighed.

5. Freeze dry the homogenate. To avoid losing leaf material during this process, remove the lid and cover the Falcon tubes with paper tissue (KimWipes) fixed with an elastic band. Place the tubes in a metal rack into the freeze drier at 0.02 bar and -80 °C for 5 days. Close the tubes with their corresponding lids immediately after removing them from the freeze drier. The dried material can be stored under vacuum in a desiccator protected from light and humidity up to 6 months.

Note: In order to avoid thawing, place the Falcon tubes in a polystyrene box containing dry ice and replace the lids by the paper tissue while the Falcon tubes are in the box.

6. Determine the plant material dry weight by weighing the closed Falcon tubes after the freeze drying process.

B. Preparation of solutions and filters

1. Prepare the solutions for the NAF three days before starting the fractionation.
2. Add the activated molecular sieve 4 Å to the Heptane (C₇H₁₆) and Tetrachloroethylene (C₂Cl₄) solutions to ensure that no residual water is present.
3. Prepare a mixture of Tetrachloroethylene/Heptane 66:34 (v/v); density = 1.3 g cm⁻³. Add the activated molecular sieve 4 Å and store the mixture protected from light in a brown glass bottle.
Note: We usually use the ~100 ml of activated molecular sieve in a 1 L bottle. For 10 samples it is needed: a) 1 L of the Tetrachloroethylene/Heptane mixture 66:34 (v/v): 660 ml of Tetrachloroethylene + 340 ml of Heptane, b) 2 L of Heptane, c) 500 ml of Tetrachloroethylene .
4. Before use always check the pH of the solutions with a pH paper, especially Tetrachloroethylene. pH should be neutral. If Tetrachloroethylene solution turns acidic, the dried leaf material will become brown during gradient centrifugation and the NAF will fail.
5. Cut 15 cm x 15 cm the nylon filters (one per NAF), weigh them and store them in clean 50 ml Falcon tubes.

C. Fractionation procedure

Note: The whole procedure should be performed under a fume hood. Only dried aliquots (after Step C17) can be processed on regular bench. For pouring solutions, use glass pipettes.

1. Resuspend the dried leaf material in 20 ml of the Tetrachloroethylene/Heptane = 66:34 (v/v) mixture.
2. Ultrasonicate the suspension for 2 min, with 6 x 10 cycles, 65% of power (Figure 2). To avoid overheating the suspension during the sonication process, keep the Falcon tube in an ice bath.
Note: Place the sonicator tip inside the solution, but avoid touching the tube wall. Make sure the

sonication is successful. This is a critical step in the fractionation, since insufficient sonication can produce aggregates that do not pass through nylon mesh.

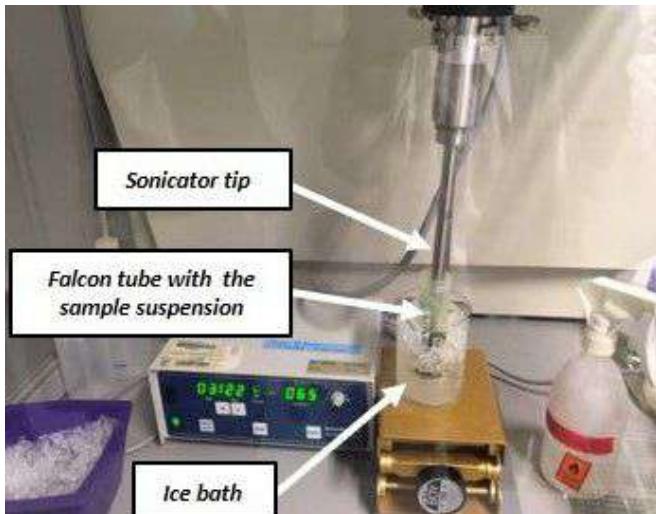


Figure 2. Setup for the ultrasonication procedure. Note that to avoid overheating the suspension during the process, the Falcon tube should be kept in an ice bath.

3. Fold a nylon mesh into a glass funnel placed on top of a 50 ml Falcon tube and pour the suspension through the nylon mesh. Wash the nylon mesh 3 x with 10 ml of heptane.
Note: The first two washing steps are performed by slowly pouring heptane on the nylon mesh and collecting the flow through in the Falcon tube below. For the third washing step, remove all the residual solution in the nylon mesh by squeezing it (while wearing adequate gloves). Place the nylon mesh back in a 50 ml Falcon tube and continue at Step C16. Be careful that all liquid go through the nylon mesh. If it's the case, filter the whole obtained mixture again through a fresh nylon mesh.
4. Centrifuge for 10 min at 3,200 $\times g$ and 4 °C, using a swing-out rotor. Discard the supernatant (by pouring it into a waste glass bottle) and resuspend the pellet in 5 ml of Tetrachloroethylene/Heptane = 66:34 (v/v) mixture.
5. Transfer 100 μl of the well homogenized resuspended pellet into a 1.5 ml screw cap tube. This aliquot is named fraction 0 (F0) and corresponds to the unfractionated material. Prepare three aliquots, add 900 μl of C₇H₁₆ to each, and keep protected from the light and continue at Step C15.
Note: Use a cut 200 μl pipette tip to collect F0. Homogenize well the mixture before taking each aliquot.
6. Prepare the NAF gradient (Figure 3A): Linear gradient (30 ml, density from 1.43 to 1.62 g cm⁻³)
 - a. With a Pasteur pipette fill the 50 ml Falcon tube A with Tetrachloroethylene/Heptane mixture and the 50 ml Falcon tube B with Tetrachloroethylene.
 - b. The dispensing needle is placed above a waste container.
Note: Ensure that there are no air bubbles in any tubing by purging thoroughly the whole

system.

- c. Set the peristaltic pump at a flow rate of 1.15 ml min^{-1} and the following program:

Step	ml	Solvent A	Solvent B
1*	0	70%	30%
2**	3	70%	30%
3	28	0%	100%
4	33	0%	100%

Notes:

- i. *After 3 ml, pause the program. This step is used to ensure that the first mixture reaches the end of the dispensing needle before starting the actual gradient.
- ii. **Remove the waste container and place the Polyallomer tube at this step. The needle should be placed slightly over the bottom of the Polyallomer tube and slightly touching the side of the tube (Figure 3B). Re-start the program.

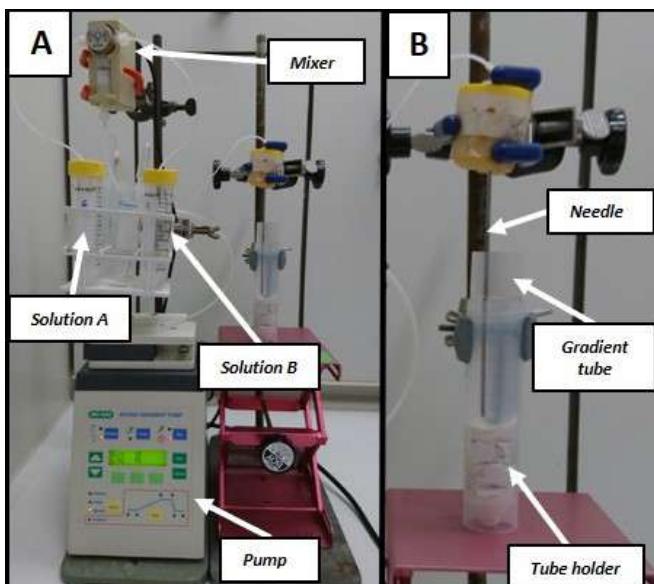


Figure 3. Setup of the gradient pump and the mixer. A. Overview of the setup for preparing the NAF gradient. Solution A (Tetrachloroethylene/Heptane mixture) and solution B (Tetrachloroethylene). B. Detail of the bottom-up preparation of the gradient. Tube holder is a 50 ml falcon tube with paper support on the bottom to help holding the gradient tube.

7. Take the needle carefully out from the gradient and place the Polyallomer tube in a rack. Apply the suspension from Step C4 to the gradient, very carefully with a Pasteur pipette. Touch the wall of the tube with the pipette slightly above the gradient and release carefully to avoid disturbing the gradient.
8. Place the gradients in the appropriate tube buckets from the ultracentrifuge. Verify their weights and to counterbalance, adjust the weight by adding 100% C_2Cl_4 .
9. Centrifuge for 50 min at $5,000 \times g$, and 4°C using a swinging bucket rotor.

Note: We always use the Ultracentrifuge Beckman Coulter Optima™ L80 XP and Swinging bucket rotor Beckman Coulter SW32Ti. Set the acceleration/deceleration on three.

10. Place the gradient tubes in a rack and mark eight fractions. Figure 4 illustrates how we usually separate the fractions based on the color.

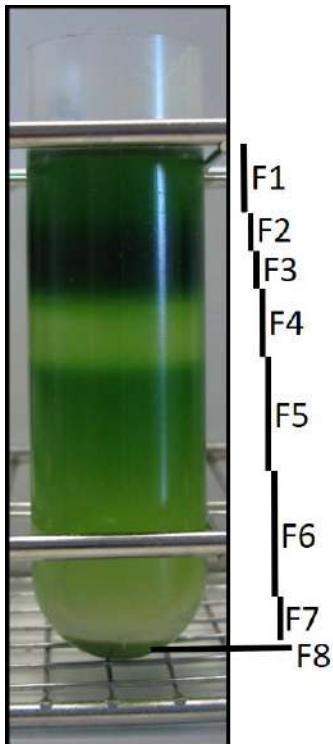


Figure 4. NAF gradient after centrifugation. Usually, we separate eight fractions based on their color. The dark green layer is enriched in chloroplast and usually divided into two fractions (F2 and F3) to avoid metabolite overloading. Note that fraction 8 is a pellet, which is the vacuolar-enriched fraction.

11. Transfer carefully each fraction into clearly labeled clean 50 ml Falcon tube by using Pasteur pipette. The fractions are collected starting from the upper part (F1) to the bottom (F8). After F7 is removed, the pellet is resuspended in 20 ml heptane and the mixture transferred to a clean 50 ml Falcon tube. Use a new pipette for each fraction.

Note: Transferring the fractions is a critical point for the method, since at this step cross contamination of the fractions should be avoided as much as possible. Therefore, always transfer the fractions from the very top of each layer.

12. Add heptane up to 20 ml to the Falcon tubes containing F1 to F8 and mix carefully. Centrifuge all Falcon tubes for 10 min at 3,200 $\times g$ and at room temperature.
13. Discard the supernatant (by carefully pouring it into a waste glass bottle) and resuspend the pellet with 7 ml of Heptane.

Note: The volume depends on how many aliquots you will need for your analysis. We usually prepare seven sub-aliquots per fraction. This number is enough for various metabolite analysis

and determination of the compartment-specific markers.

14. Transfer 6 x 1 ml from each suspension into 1.5 ml screw caps tubes. The pellet can easily re-aggregate at this step. Keep mixing by hand the solution while transferring into the tubes in order to obtain homogeneous aliquots from the same fraction.

Note: Use a cut 1000 µl pipette tip to collect sub-aliquots from each fraction. As adding 7 ml generally does not allow to obtain seven aliquots of 1 ml, we generally take 0.8 ml for the seventh aliquot and clearly label the corresponding tube.

15. Centrifuge for 10 min at 14,000 x g and at room temperature. Discard excess of supernatant (c.a. 900 µl) carefully, using a 1000 µl pipette tip. Process the F0 aliquots obtained in Step C5 in the same manner.
16. Place the screw caps tubes containing the sub-aliquots open in racks inside of a desiccator connected to a vacuum pump. Also, place the open Falcon tubes containing the filters from Step C3. Keep the aliquots under vacuum and protected from light overnight.
17. Close the tubes and store them at -80 °C until further process.
18. Weigh the dried filters.

D. Measurements of metabolite markers

1. Extraction

- a. Add to the dried aliquots a steel ball and 250 µl 80% ethanol/10 mM HEPES pH 7 to the samples.
- b. Place the tubes in a cooled (4 °C) holder for the ball mill and disrupt the pellet for 1 min at 25 Hz.
- c. Incubate in a thermomixer for 30 min at 95 °C and 1000 rpm.
- d. Centrifuge for 10 min at 20,800 x g and 4 °C.
- e. Transfer the supernatant to a 96-deep well plate and keep it protected from light and on ice.
- f. Add to the Pellet 150 µl 80% ethanol/10 mM HEPES pH 7.
- g. Incubate in a thermomixer for 30 min at 95 °C 1000 rpm.
- h. Centrifuge for 10 min at 20,800 x g and 4 °C.
- i. Transfer and combine with the previous supernatant.
- j. Add to the Pellet 250 µl 50% ethanol/10 mM HEPES pH 7.
- k. Incubate in a thermomixer for 30 min at 95 °C and 1,000 rpm.
- l. Centrifuge for 10 min at 20,800 x g and 4 °C.
- m. Transfer and combine with the previous two supernatants. It can be stored at -20 °C until further use.

2. Nitrate (vacuolar marker)

Nitrate content is determined following Cross *et al.* (2006). Nitrate can be used as a vacuolar marker in addition or alternatively to Acid Invertase.

Note: Nitrite content must be determined and subtracted from the Nitrate values. For measuring nitrite content, nitrate reductase is replaced by water in the assay mix.

Solutions: For storage recommendations, the solutions for nitrate measurement can be stored as follow:

Solution:

	Storage temperature
Potassium phosphate buffer 1 M pH 7.5	-20 °C
NADPH 50 mM in NaOH 20 mM	-80 °C
Nitrate reductase (5 U ml ⁻¹ in Potassium phosphate buffer 0.1 M)	-80 °C
PES 0.25 mM	-80 °C
Sulfanilamide 1% (w/v) in phosphoric acid 5%	4 °C
NNEDA 0.02% (w/v)	4 °C
Nitrate standards	-80 °C
Nitrate Standards: 0, 0.2, 0.4, 0.8, and 1.6 mM in 70% ETOH with 10 mM MES pH 5.9	

Protocol:

- For one reaction, dispense in a 96-well plate 95 µl of the assay mix:

10.0 µl phosphate buffer
0.5 µl NADPH
2.0 µl Nitrate reductase
82.5 µl deionized water

- Add 5 µl of standard or extract (1 to 10 diluted extract).
- Mix and incubate for 30 min at 25 °C, protected from light.
- Add 15 µl of PES.
- Mix and incubate for 20 min at 25 °C, protected from light.
- Add 50 µl of Sulphanilamide and 50 µl of NNEDA.
- Mix and incubate for 10 min at 25 °C, protected from light.
- Read the plate at 540 nm in a microplate reader.

E. Measurements of enzyme markers

1. Enzyme extraction

- Add to the dried extract ~2 mg of PVPP (one little spoon), one steel ball (5 mm diameter), and 500 µl of buffer 1x (see Recipe 1b). Keep the samples on ice.
- Place the tubes in a cooled (4 °C) holder for the ball mill and disrupt the pellet for 1 min at 25 Hz. Make sure that the entire pellet is dissolved. If not, repeat the pellet disruption procedure.
- Centrifuge for 10 min at 20,800 x g and 4 °C. Transfer the enzyme extract (supernatant) to a 96 deep-well plate. Keep on ice and proceed to the enzyme assays. If required, the supernatant can be stored at -80 °C.

2. Enzyme assays

All the three enzyme markers are measured according to Gibon et al. (2004).

a. AGPase activity (plastid marker)

- Dispense in a 96-well plate placed on ice:

- 14 µl of AGPase assay mix (see Recipe B2b)
- 6 µl of standard or extract.
- ii. Mix and incubate for 20 min at 25 °C.
 - iii. Add 20 µl of 0.5 M HCl in 100 mM Tricine/KOH pH 8.
 - iv. Mix and incubate for 10 min at room temperature.
 - v. Add 20 µl of 0.5 M NaOH.
 - vi. Mix, add 50 µl of AGPase Determination mix (see Recipe B2c).
 - vii. Mix, spin down, and read at wavelength of 340 nm and 30 °C until the maximum rate of reaction stabilizes.
- b. PEPC (Cytosol marker)
- i. Dispense in a 96-well plate placed on ice.
- 18 µl PEPC Assay Mix (see Recipe B3b)
- 2 µl of standard or extract
- ii. Mix and incubate for 20 min at 25 °C.
 - iii. Add 20 µl of 0.5 M HCl in 100 mM Tricine/KOH pH 9.
 - iv. Mix, spin down, and incubate at 95 °C for 5 min. Cool and spin down. Keep the plate on ice.
 - v. Add 20 µl 0.5 M NaOH.
 - vi. Mix, add 45 µl of PEPC Determination mix (see Recipe 3c, protect from light).
 - vii. Add 5 µl of 4 mM PES (protect from light).
 - viii. Mix and read at wavelength of 570 nm and 30 °C until the maximum rate of reaction stabilizes.
- c. Acid Invertase (Vacuole marker)
- i. Dispense in a black 96-well microplate:
- 10 µl Acid invertase Assay mix (see Recipe B4b)
- 5 µl of standard or enzyme extract
- ii. Mix and incubate at 25 °C for 5 and 40 min.
 - iii. Add 10 µl 0.5 M NaOH.
 - iv. Mix and incubate at R.T. for 10 min.
 - v. Add 10 µl 0.5 M HCl in 100 mM Tricine/KOH pH 8.
 - vi. Mix and add 50 µl of Acid invertase Determination mix (see Recipe B4c).
 - vii. Mix and read the fluorescence for 10-15 min using the following setting:
Excitation at 530 nm, emission at 590 nm, temperature: 30 °C, sensitivity at 25. Use the rate of reaction for calculations. We use the Synergy fluorescence meter.

Data analysis

1. For evaluation of subcellular metabolite distributions, we recommend the statistical software BestFit. The BestFit is a C-language command line tool that allows calculation and evaluation

of subcellular distributions from NAF data. The software requires; (i) the distribution of specific markers for each subcellular compartment analyzed throughout the different fractions (Figure 5) and (ii) the distribution of the metabolites of interest throughout the different fractions.

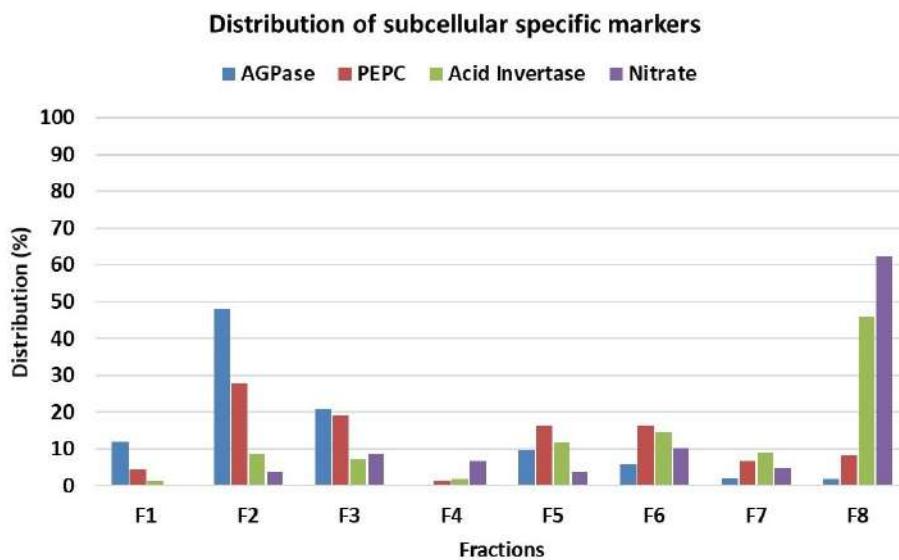


Figure 5. Distribution in percentage of subcellular compartment-specific markers throughout the gradient showed in Figure 4. ADP glucose pyrophosphorylase (AGPase) as plastidic marker, phosphoenolpyruvate carboxylase (PEPC) as cytosolic marker and nitrate and acid invertase as vacuolar markers.

2. For the values of either markers and metabolites of interest in the input file, we recommend using percentage, although absolute measurements can also be used (note that in this case values must be ≥ 0).
3. For more details on how to use BestFit, we suggest reading the documentation pdf file within the BestFit Folder (<http://www.csbdb.de/csbdb/bestfit/bestfit.html>). Alternatively, you can also find details regarding data input and use of BestFit in Krueger et al. (2014).
4. BestFit outputs the subcellular-distribution of the metabolites of interest in percentage. In order to calculate which amounts this represents, metabolites are also measured in ground material not processed through NAF (see Note in A1). Various recovery calculations can be done by using the following information: (i) material fresh weight used for NAF (determined in step A4), (ii) corresponding dry weight (determined in step A6), (iii) how much material was applied to the gradient (determined in step C18 by weighing the residual material in the filter), (iv) F0 obtained step C6. We recommend always recording these data and collecting F0 for each NAF.

Recipes

A. Measurement of metabolite markers

1. Nitrate standards

0, 0.2, 0.4, 0.8, and 1.6 mM in 70% ETOH with 10 mM MES pH 5.9

B. Measurement of enzyme markers

1. Extraction buffers

a. Extraction Buffer 10x

500 mM HEPES

100 mM MgCl₂

10 mM EDTA

10 mM EGTA

10 mM Benzamidine

10 mM ε-aminocapronic acid

2.5% w/v BSA (protease-free)

Bring volume to c.a. 40 ml, adjust pH to 7.5 with KOH 10 M, and then adjust the volume to 50 ml

The extraction buffer 10x can be stored at -20 °C

Prepare the following solutions and store accordingly:

Leupeptin 2 mM (100x), freeze in liquid nitrogen and then store at -80 °C

DTT 500 mM (1,000x), freeze in liquid nitrogen and then store at -80 °C

PMSF 100 mM in isopropanol (100x), store at 4 °C, protect from light, stable for 1 month

Triton X-100 10% v/v (10x), store at RT

Glycerol (87% v/v), store at RT

b. Extraction Buffer 1x

Prepare only prior the extraction combining as the following:

[Final]	Reagent	[stock]	For 1,000 µl
1x	Buffer 10x (see Recipe 1a)	10x	100 µl
10 µM	Leupeptin	2 mM	5 µl
1 mM	DTT	500 mM	2 µl
1 mM	PMSF	100 mM in isopropanol	10 µl
1%	Triton X-100	10% v/v	100 µl
20%	Glycerol	87% (v/v)	230 µl
	H ₂ O		553 µl

2. AGPase activity

a. AGPase assay buffer (can be stored at -20 °C)

0.25 M HEPES/KOH pH 7.5

7.5 mM NaF

25 mM MgCl₂

5 mM 3-phosphoglycerate

0.25% (v/v) Triton X-100

b. AGPase assay mix

- 7.6 μ l water
4 μ l AGPase assay buffer (see Recipes B2a)
2 μ l 20 mM PPi
0.2 μ l Glycerokinase (200 U ml⁻¹ in 200 mM Tricine/KOH pH 8, 10 mM MgCl₂)
0.2 μ l ADP-glucose 0 mM (Blank) or 100 mM (maximum)
- c. AGPase determination mix
37.8 μ l water
10 μ l 1 M Tricine/KOH pH 8
0.2 μ l 1 M MgCl₂
0.5 μ l GDH (200 U ml⁻¹ in 200 mM Tricine/KOH pH 8, 10 mM MgCl₂)
0.5 μ l GPOX (500 U ml⁻¹ in 200 mM Tricine/KOH pH 8, 10 mM MgCl₂)
1 μ l 66 mM NADH
3. PEPC activity
- a. PEPC assay Buffer (can be stored at -20 °C)
0.5 M Tricine/KOH pH 8
100 mM MgCl₂
50 mM NaHCO₃
0.25% Triton X-100
- b. PEPC assay Mix
11.4 μ l H₂O
4 μ l PEPC assay Buffer (see Recipe B3a)
2 μ l 0 (Blank) or 20 mM PEP (maximum)
0.4 μ l 5 mM NADH
0.2 μ l Malate dehydrogenase (100 U ml⁻¹ in 200 mM Tricine/KOH pH 8, 10 mM MgCl₂)
- c. PEPC determination mix (protect from light)
18 μ l water
10 μ l 1M Tricine/KOH pH 9.0
10 μ l 10 mM MTT
4 μ l 200 mM EDTA
2 μ l 50% Ethanol
1 μ l Alcohol Dehydrogenase (2,000 U ml⁻¹ in 200 mM Tricine/KOH pH 9, 10 mM MgCl₂)
4. Acid Invertase activity
- a. Acid invertase assay Buffer (can be stored at -20 °C)
0.25 M Acetate/KOH pH 5
- b. Acid invertase Assay Mix: for 1 reaction
10 μ l Acid invertase assay buffer (see Recipe B4a)
35 μ l water
- c. Acid invertase Determination mix (protect from light): for 1 reaction
42 μ l water

5 µl 1 M HEPES/KOH pH 7
1 µl Glucose oxidase (200 U ml⁻¹ in 200 mM HEPES/KOH pH 7.0)
1 µl Horse Radish Peroxidase (0.2 U ml⁻¹ in 200 mM HEPES/KOH pH 7.0)
1 µl 20 mM Amplex Red in DMSO

Acknowledgments

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We also would like to thank the authors from Krueger et al. (2014), the previous work describing the NAF method, which we made the modification presented in the present protocol.

Competing interests

The authors declare no conflict of interest.

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Cell Wall Compositional Analysis of Rice Culms

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[Abstract] The plant cell wall is a complicated network that is mainly constituted of polysaccharides, such as cellulose, hemicellulose and pectin. Many noncellulosic polysaccharides are further acetylated, which confers these polymers flexible physicochemical properties. Due to the significance of cell wall in plant growth and development, the analytic platform has been the focus for a long time. Here, we use internodes/culms, an important organ to provide mechanical support for rice plants, as an experimental sample to explore the method for cell wall composition analysis. The method includes preparation of cell wall residues, sequential extraction of polysaccharides, and measurement of cellulose. The procedure for acetate examination is also described. This method is applicable to determine the composition of individual cell wall polymers and the modifier acetates, and is suitable to identify cell wall relevant mutants based on the advantages in high throughput, precision and repeatability.

Keywords: Xylan, Pectin, Cellulose, Acetylation, Cell wall, Rice

[Background] The plant cell wall represents one of the most complicated cellular structure in nature and is essential for plant growth and adaptations to environments. Besides presenting multiple polysaccharide components and phenolic compounds, acetylation is a prevalent modification on most cell-wall polymers, which alters the physicochemical properties and increases the complexity of cell wall structure. Establishment of the effective analytic platform for cell wall composition is always a challenging task. The previous analytic method often uses alkali to extract cell wall residues, but removes acetate. Recent works have revealed that acetate patterns on xylan determine the folding of this polymer and impact the binding to cellulose or lignin, indicating its importance in cell wall formation and plant growth control (Grantham et al., 2017; Kang et al., 2019; Zhang et al., 2019). The method that can simultaneously examine the composition of diverse cell-wall polysaccharides and their acetyl modifications needs to be developed. It becomes realizable as solvent dimethyl sulfoxide has been found extracting xylan without trimming acetyl esters (Goncalves et al., 2008). Rice culms are representative for cell wall composition methodology analysis because this organ is rich of secondary wall-bearing fiber cells and also contains multiple cell types. In addition to abundant materials, acetylation level varies on different wall polymers and during the culm development. By using rice culms as analytic samples, we developed a protocol for cell wall composition and acetyl modification analyses with some changes from the previous method (Foster et al., 2010). This protocol offers a widely used way to examine the composition of diverse cell wall polymers and determine the acetate content in

different rice varieties and other crops.

Materials and Reagents

1. 96-well flat bottom assay plate (Greiner bio-one, catalog number: 655180)
2. UV capable 96-well flat bottom assay plate (Corning, catalog number: 3635)
3. Glass bottle
4. Eppendorf tubes (1.5 ml) (Eppendorf, catalog number: 0030120.086)
5. Sarstedt tubes 2 ml (Sarstedt, D-51588)
6. 50 ml plastic centrifuge tube (Corning CentriStar)
7. Glass microfiber filters (Whatman, catalog number: 1820-025)
8. Rice mature plants
9. Endopolygalacturonase M2 (Megazyme, catalog number: PGALUSP, 4 °C)
10. Pectin methyl esterase (Sigma-Aldrich, catalog number: P5400-1KU, -20 °C)
11. α-amylase (Megazyme, catalog number: E-BLAAM, 4 °C)
12. ddH₂O
13. Acetatic Acid Assay Kit (Megazyme, catalog number: K-ACET, 4 °C)
14. Acetone
15. DMSO (Sigma-Aldrich, catalog number: D5879)
16. 70% (v/v) aqueous ethanol
17. Chloroform/methanol (1:1, v/v) solution
18. Updegraff reagent (Acetic acid: nitric acid: water, 8:1:2 v/v)
19. 72% Sulfuric acid (Prepared with concentrated Sulfuric acid GR)
20. 1 mg/ml glucose stock (Prepared from D-(+)-glucose) (Sigma-Aldrich, catalog number: G8270, -20 °C)
21. Anthrone reagent (2 mg/ml Anthrone in concentrated sulfuric acid) (Sigma-Aldrich, catalog number: 319899)
22. Trifluoroacetic acid (TFA) (Sigma-Aldrich, catalog number: T6508)
23. Ammonium formate (Aldrich, catalog number: 516961)
24. 11% peracetic acid solution (prepared from 35% peracetic) (Aladdin, catalog number: P112625)
25. Ethanol: methanol: water solution (7:2:1, adjust the pH to 3.0 with HCOOH)
26. 1% ammonium oxalate (Sigma-Aldrich, catalog number: 09898)
27. 37% hydrogen chloride
28. MES/Tris buffer (pH 8.1-8.3) (see Recipes)
29. 2 M trifluoroacetic acid (see Recipes)
30. 1 N sodium hydroxide (see Recipes)
31. 1 N hydrogen chloride (see Recipes)
32. 50 mM ammonium formate (pH 4.5) (see Recipes)

Equipment

1. Freeze dryer (Beijing Songyuanhuaxing Technology Develop Co. Ltd., model: LGJ-12)
2. Ball mill (QIAGEN, TissueLyser II, catalog number: 85300)
3. (Optional) Vortex shaker
4. Basket centrifuge (Eppendorf, model: 5810R)
5. Centrifuge (Eppendorf, model: 5430) (to fit Eppendorf 1.5 ml tubes)
6. Thermomixer comfort (Eppendorf)
7. Dri-Block heaters (Techne, model: DB200/3)
8. Microplate reader (PerkinElmer, Enspire)
9. Concentrator (Eppendorf, concentrator plus)
10. (Optional) Drying oven
11. (Optional) Shaking incubator
12. Sieves (Mesh size of 0.15 mm)
13. pH meter (Mettler Toledo)
14. Semi-micro scales (dual resolutions starting at 0.01 mg)

Procedure

- A. Preparation of destarched alcohol-insoluble cell-wall residues (AIR)
1. Pool the whole 2nd internodes (numbered from the top down) of 5-20 rice mature plants.
 2. Freeze the fresh samples in liquid nitrogen and then lyophilize them in a freeze dryer (The rice internodes were lyophilized for 48 h to ensure complete dryness).
 3. Grind tissues to a particle size no more than 0.15 mm using ball mill and sieve through mesh with a size of 0.15 mm.
 4. Weigh approximately 1 g of the ground plant biomass into a 50 ml plastic centrifuge tube.
 5. Add 30 ml of 70% (v/v) aqueous ethanol, mix thoroughly using a vortex mixer and leave in a thermomixer comfort set at 37 °C and 200 rpm for 12 h.
 6. Centrifuge at 1,500 $\times g$ for 10 min in a basket centrifuge and discard the supernatant.
 7. Repeat Steps A5-A6 once.
 8. Add 30 ml of the chloroform/methanol (1:1 v/v) solution, mix thoroughly using a vortex mixer and leave in a shaking incubator for 30 min at 37 °C and 200 rpm.
 9. Centrifuge at 1,500 $\times g$ for 10 min at room temperature and discard the supernatant.
 10. Repeat Steps A8-A9 twice.
 11. Add 15 ml of acetone, shake the tube to re-suspend the pellet.
 12. Centrifuge at 1,500 $\times g$ for 10 min and discard the supernatant.
 13. Repeat Steps A11-A12 twice.
 14. Let the biomass samples dry in a drying oven at 40 °C without shaking for approximately 16 h.
 15. Treat the residues with 100 U α-amylase in 40 ml MES/Tris buffer (pH 8.1) at 97 °C for 35 min,

then 60 °C for 1 h.

16. Centrifuge at 1,500 $\times g$ for 10 min and discard the supernatant.
17. Wash the pellet with 30 ml ddH₂O three times and with 15 ml acetone twice, with centrifugation (1,500 $\times g$ for 10 min) and supernatant removal after each wash.
18. Let the biomass samples dry in an oven at 40 °C for approximately 16 h to get destarched AIR.

B. Analysis of the crystalline cellulose content

1. Weigh 2 mg AIR material in five replicates into 2 ml Sarstedt tubes.
2. Add 250 μ l of 2 M trifluoroacetic acid (TFA) to each sample and make ensure no material is splashed up onto the tube walls.
3. Cap tightly and incubate for 90 min at 121 °C in Dri-Block heaters.
4. Cool the heating blocks and samples on ice.
5. Centrifuge at 11,000 $\times g$ for 10 min, then transfer the supernatant to a new Sarstedt tube for optional noncellulosic polysaccharides composition analysis (Foster *et al.*, 2010) and keep the pellet for crystalline cellulose assay.
6. Add 1 ml of Updegraff reagent (Acetic acid: citric acid: water, 8:1:2 v/v) to the pellets left over from the Step B5, cap tightly and vortex.
7. Heat in Dri-Block heaters at 100 °C for 30 min.
8. Cool samples on ice.
9. Centrifuge samples at 11,000 $\times g$ for 10 min.
10. Discard supernatant ensuring no pellet material is discarded.
11. Wash once with 1 ml water and four times with 1 ml acetone, centrifuge and discard supernatant as done above.
12. Air dry the pellet in the Dri-Block heaters at 35 °C.
13. Add 175 μ l 72% Sulfuric acid and incubate at room temperature for 60 min.
14. Add 825 μ l ddH₂O, vortex and centrifuge samples at 11,000 $\times g$ for 5 min.
15. Analyze the glucose content of the supernatant using the anthrone assay in a 96-well flat bottom assay plate.
16. Add 10 μ l of sample and 90 μ l of ddH₂O for a total volume of 100 μ l in each sample well.
17. Prepare standards using 1 mg/ml glucose stock (stock at -20 °C). Make 0, 2, 4, 6, 8, and 10 μ g standards by pipetting 0, 2, 4, 6, 8, and 10 μ l into the appropriate well, adding 100, 98, 96, 94, 92, and 90 μ l ddH₂O accordingly.
18. Add 200 μ l freshly prepared Anthrone Reagent.
19. Heat the plate for 30 min at 80 °C with shaking in a thermomixer comfort. Cool to room temperature.
20. Shake the plate thoroughly in a thermomixer comfort and then read absorption at 625 nm within 1 h.

C. Extraction of pectin from cell wall residues

1. Weigh about 6 mg destarched AIR for 5 replicates into 1.5 ml Eppendorf tubes.
2. Add 1 ml of 50 mM ammonium formate (pH 4.5) buffer, then add 2 U of endopolygalacturonase M2 and 0.04 U of pectin methyl esterase. Mix and incubate at 37 °C for 16 h with shaking at 300 rpm in a thermomixer comfort.
3. Centrifuge at 3,000 $\times g$ for 10 min and transfer the pectin-rich supernatants to a new tube. Keep the remains as pectin-free samples.
4. Freeze the supernatants using liquid nitrogen and then lyophilize them in a freeze dryer, finally get the gelatinous pectin.

D. Isolation of the acetyl-xylan from AIR

1. Weigh about 400 mg of destarched AIR into a 50 ml centrifuge tube.
2. Add 30 ml 1% ammonium oxalate and incubate at 85 °C for 2 h. Centrifuge at 1,500 $\times g$ for 15 min in a basket centrifuge. Discard the supernatant to remove pectin.
3. Add 30 ml 11% peracetic acid solution, incubate in a water bath at 85 °C for 30 min for delignification.
4. Centrifuge at 1,500 $\times g$ for 10 min. Discard the supernatant and wash the pellets with 30 ml ddH₂O three times and with 15 ml acetone once, through centrifugation (1,500 $\times g$ for 10 min) and supernatant removal.
5. Let the pellets dry in an oven set at 40 °C for approximately 16 h.
6. Add 30 ml DMSO and then incubate at 70 °C for 12 h to conduct extraction, centrifuge at 1,500 $\times g$ for 10 min, transfer the supernatant to a new glass bottle.
7. Repeat Step D6 with 12 ml DMSO, and combine the supernatants containing the extractives.
8. Filter the extracts through glass microfiber filters.
9. Precipitate the extracts with 5 volume of ethanol: methanol: water solution (7:2:1, pH 3.0) at 4 °C for 12 h.
10. Centrifuge at 1,500 $\times g$ for 15 min. Discard the supernatant and wash the pellets four times each with 10 ml anhydrous ethanol with centrifugation (1,500 $\times g$ for 10 min) and supernatant removal after each wash.
11. Dry the pellets under vacuum in a concentrator at room temperature.

E. Determining the content of acetyl esters

1. Weigh about 1 mg destarched AIRs/pectin/xylan for 5 replicates into 1.5 ml Eppendorf tubes.
2. Add 100 μ l 1 N sodium hydroxide to tubes and shake for 1 h at 28 °C and 200 rpm to release the bound acetate.
3. Add 100 μ l of 1 N hydrogen chloride to neutralize samples.
4. Centrifuge at 15,000 $\times g$ for 10 min, and transfer 10 μ l supernatant aliquot to a UV capable 96-well flat bottom assay plate and immediately quantify released acetic acids according to the instruction of Acetic Acid Assay Kit.

5. Add 94 μ l of ddH₂O to each sample well in the UV capable 96-well flat bottom assay plate.
6. Add 42 μ l of freshly prepared mixture of kit-supplied Solutions 1 and 2 (2.5:1, 30 μ l + 12 μ l each), mix and incubate at 25 °C for 3 min with shaking at 300 rpm in a thermomixer comfort.
7. Read the absorption at 340 nm (A0).
8. Add 12 μ l of freshly diluted kit-supplied solution 3 (1:10, v/v, in ddH₂O), mix and incubate at 25 °C for 4 min with shaking at 300 rpm in a thermomixer comfort.
9. Read the absorption at 340 nm (A1).
10. Add 12 μ l of freshly diluted kit-supplied solution 4 (1:10, v/v, in ddH₂O), mix and incubate at 25 °C for 12 min with shaking at 300 rpm in a thermomixer comfort.
11. Read the absorption at 340 nm (A2).
12. At the same time, it is necessary to make a blank control and a standard curve in parallel. To make a standard curve, add 5, 10, 15, 30, 50 μ l acetic acid standard (solution 5) equal to 0.5, 1, 1.5, 3, 5 μ g, and adjust the volume of ddH₂O in Step E5 to 99, 94, 89, 74, 54 μ l, respectively.

Data analysis

1. Generation of standard curve and calculation of cellulose content

To calculate cellulose amount, the standard curve needs to be plotted out first (The absorbance of glucose standards versus the concentration of glucose standards). Then calculate cellulose content (μ g glucose/mg AIR) of sample with standard curve.

2. For acetylation content analysis: Δ acetic acid = $(A_2 - A_0)_{\text{sample}} - (A_1 - A_0)^2_{\text{sample}} / (A_2 - A_0)_{\text{sample}} - [(A_2 - A_0)_{\text{blank}} - (A_1 - A_0)^2_{\text{blank}} / (A_2 - A_0)_{\text{blank}}]$.

Locate the sample value in the standard curve, and get acetic acid content in the sample aliquot. Calculate sample contents by multiplying with a factor of (100 + 100)/10/weight (μ g/mg AIR).

3. For statistical analysis, typically use at least five biological replicates from a pool of internodes. Results are reported as means and standard deviation and statistical significance is assessed by Student's *t*-test or one-way ANOVA followed by Tukey's multiple comparison test.

Notes

1. If a freeze-dryer is not available, it could be alternative to prepare AIR directly from frozen tissue that is homogenized with a mortar and pestle immersed in liquid nitrogen or a ball mill pre-immersed in liquid nitrogen.
2. For acetylation analysis, the acetic acid released should be assayed as soon as possible, do not leave too long before plate reading.

Recipes

1. MES/Tris buffer (pH 8.1)

- a. Add 488 mg MES and 355 mg Tris in 40 ml double distilled water
- b. Adjust the pH to 8.1 with sodium hydroxide and finally dilute to 50 ml
2. 2 M trifluoroacetic acid
Dilute 15.31 ml TFA with ddH₂O to a final volume of 100 ml
3. 1 N sodium hydroxide
Add 2 g of sodium hydroxide to 50 ml of ddH₂O
4. 1 N hydrogen chloride
Mix 4 ml 37% hydrogen chloride with 44 ml ddH₂O
5. 50 mM ammonium formate (pH 4.5)
 - a. Add 0.15 g of ammonium formate to 50 ml ddH₂O
 - b. Adjust the pH to 4.5 with formic acid

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Competing interests

The authors declare no conflict of interest.

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Visualization of Actin Organization and Quantification in Fixed *Arabidopsis* Pollen Grains and Tubes

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[Abstract] Although it is widely accepted that actin plays an important role in regulating pollen germination and pollen tube growth, how actin exactly performs functions remains incompletely understood. As the function of actin is dictated by its spatial organization, it is the key to reveal how exactly actin distributes in space in pollen cells. Here we describe the protocol of revealing and quantifying the spatial organization of actin using fluorescent phalloidin-staining in fixed *Arabidopsis* pollen grains and pollen tubes. We also introduce the method of assessing the stability and/or turnover rate of actin filaments in pollen cells using the treatment of latrunculin B.

Keywords: *Arabidopsis*, Pollen germination, Pollen grain, Pollen tube, Actin, Phalloidin staining

[Background] Pollen germination and subsequent pollen tube growth is the critical step during sexual plant reproduction, as it provides passage for the delivery of two non-motile sperm cells to finally effect double fertilization in flowering plants (McCormick, 2013). How pollen germination and pollen tube growth are regulated has been subject to intensive scrutiny in the past several decades. It was shown that actin is an essential regulator of pollen germination and pollen tube growth and morphogenesis (Gibbon et al., 1999; Vidali et al., 2001). As pollen germination and pollen tube growth are very sensitive to the perturbation on the actin cytoskeleton (Gibbon et al., 1999; Vidali et al., 2001), and considering along with the fact that *Arabidopsis thaliana* is amenable to genetic analysis, *Arabidopsis* pollen has increasingly becoming the great cellular system in documenting the organization and regulation of the actin cytoskeleton toward understanding how actin performs functions in pollen (Qu et al., 2015). *Arabidopsis* pollen cell is an excellent cellular system to study the organization and regulation of the actin cytoskeleton is also because the spatial organization of actin is very regular in pollen cells (Ren and Xiang, 2007; Cheung and Wu, 2008; Chen et al., 2009; Staiger et al., 2010; Fu, 2015; Qu et al., 2015), and it is therefore comparatively easy to quantify the organization of actin in pollen cells (Fu, 2015; Qu et al., 2015). The colleagues in this field have developed different methods in revealing the organization of actin in pollen cells, including staining with fluorescent-phalloidin or immune-staining probed with anti-actin antibody in fixed *Arabidopsis* pollen cells as well as decoration with different actin markers in living pollen cells. Compared to immuno-staining with anti-actin antibody in fixed pollen cells, actin staining with fluorescent phalloidin can yield more cleanly filamentous images as phalloidin specifically binds to actin filaments with high affinity that reduces the background noise, which will

facilitate subsequent analysis and quantification of actin organization in pollen cells. In addition, the procedure of actin staining with fluorescent phalloidin is comparatively simple and straightforward. Furthermore, the whole procedure was completed on solid pollen germination medium, which minimizes the effect of various treatments on the morphology of pollen tubes. This method has been carried out routinely and successfully in our laboratory. The detailed method can be found in series of our previously published papers (Wu *et al.*, 2010; Zhang *et al.*, 2010; Qu *et al.*, 2013; Zheng *et al.*, 2013; Chang and Huang, 2015; Liu *et al.*, 2015; Zhang *et al.*, 2016; Jiang *et al.*, 2017; Qu *et al.*, 2017; Lan *et al.*, 2018; Diao *et al.*, 2019; Jiang *et al.*, 2019) and the papers from our colleagues in this community (Gebert *et al.*, 2008; Cao *et al.*, 2013; Jia *et al.*, 2013; Zhu *et al.*, 2017).

Materials and Reagents

A. Materials

1. 12 cm x 12 cm square Petri dish
2. 9 cm x 9 cm glass Petri dish (NORMAX, catalog number: 5058546)
3. Microscope slide
4. Parafilm (Merck, catalog number: P7668)
5. Glass bottom dish (In Vitro Scientific, catalog number: D35-20-1-N)

B. Plant materials: *Arabidopsis thaliana* Columbia-0 (Col-0)

Grow plants in culture room under 16 h-light/8 h-dark photoperiod at 22 °C (turn on light at 7:00 am and turn off light at 11:00 pm). Choose plants in the best flower production stage (*i.e.*, choose plants with over 5 siliques on the main inflorescence stem, and don't choose older plant with few flower buds) (Figure 1A).

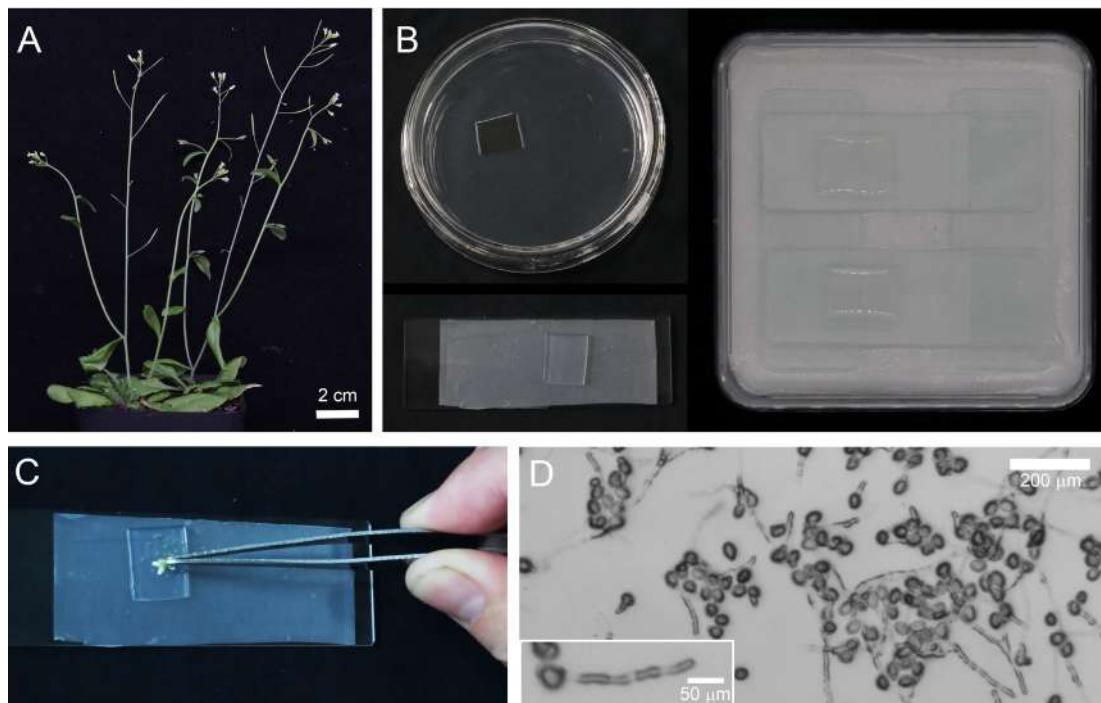


Figure 1. The Procedure for Germinating *Arabidopsis* Pollen on Solid Pollen Germination Medium. A. 6-8 weeks old *Arabidopsis* with flowers ready for the collection of pollen. Scale bar = 2 cm. B. Preparation of pollen germination medium and humid dish for pollen germination. C. Gently dip flowers to spread pollen on the surface of solid pollen germination medium. D. Images of pollen tubes after culture for 2 h at 28 °C. Scale bar = 200 μ m. Inset is a pollen tube with the length reaches at ~180 μ m. Scale bar = 50 μ m. Pollen tubes are ready for subsequent fixation and staining with phalloidin.

C. Chemical reagents

1. Calcium chloride (Merck/Sigma-Aldrich, catalog number: C7902)
2. Calcium nitrate (Merck/Sigma-Aldrich, catalog number: C1396)
3. Magnesium sulfate (Merck/Sigma-Aldrich, catalog number: M1880)
4. Boric acid (Merck/Sigma-Aldrich, catalog number: B6768)
5. Sucrose (Merck/Sigma-Aldrich, catalog number: V900116)
6. Agarose (Merck/Sigma-Aldrich, catalog number: V900510)
7. Potassium hydroxide (Merck/Sigma-Aldrich, catalog number: 484016)
8. LatrunculinB (LatB) (Merck/Calbiochem, catalog number: 428020)
9. Dimethyl sulfoxide (DMSO) (Merck/Sigma-Aldrich, catalog number: D2438)
10. 3-Maleimidobenzoic acid N-hydroxysuccinimide ester (MBS) (Merck/Sigma-Aldrich, catalog number: M2786)
11. Trizma (Merck/Calbiochem, catalog number: V900483)
12. Sodium chloride (Merck/Sigma-Aldrich, catalog number: V900058)
13. Nonidet P-40 (NP-40) (IGEPAL CA-630) (Merck/Sigma-Aldrich, catalog number: I-8896)
14. Alexa Fluor 488 phalloidin (Thermo/Invitrogen, catalog number: A12379)

15. Methanol (Merck/Sigma-Aldrich, catalog number: 494437)
16. Milli-Q water (Merck)
17. Pollen germination medium (see Recipes)
18. Tris Buffered Saline (TBS) (see Recipes)
19. Stock solution (see Recipes)
20. Individual stocks with Milli-Q water (see Recipes)

Equipment

1. Olympus FV1000S or an equivalent confocal microscope
2. Olympus CX23 or an equivalent microscope
3. Yiheng GHP-9050 or an equivalent constant temperature incubator
4. 4 °C and -20 °C freezer

Software

1. ImageJ (<https://imagej.nih.gov/ij/> version 1.52n)
2. R program (<http://www.r-project.org>)

Procedure

A. Preparation of *Arabidopsis* pollen germination medium (Figure 1B)

1. Prepare individual stocks with Milli-Q water (Recipe 1).
2. Prepare 100 ml *Arabidopsis* liquid pollen germination medium (PGM) (Recipe 2). Transfer 30 ml liquid PGM into another conical flask to prepare solid PGM. Melting agarose with microwave, and evenly spread the medium into two or three 9 cm x 9 cm glass Petri dishes.

Notes:

- a. pH at 6.85-7.0 works. Avoid adding excess KOH.
 - b. PGM can be used within 3 days under 4 °C. Old PGM will dramatically reduce germination rate.
 - c. Remember to record the weight of the PGM before and after melting agarose. The amount of evaporated water can be quantified by the difference between the two records. Add Milli-Q water back to substitute the evaporated water.
 - d. Make sure the cooling surface for placing the Petri dishes is horizontal.
3. Cut solid PGM into 1 cm x 1 cm pieces. Place the medium pieces onto parafilm, and move medium pieces and parafilm together onto microscope slide.
 4. Put the microscope slide into humid 12 cm x 12 cm square Petri dish. Place two microscope slides below to avoid water contamination.

B. Pollen collection and pollen germination

1. Harvest fresh opened *Arabidopsis* flowers during the period from 9:30 am to 12:00 pm and leave them at room temperature. Store no longer than 1 h.
2. Gently dipping the *Arabidopsis* flower onto the medium to spread the pollen (Figure 1C). For the observation of pollen grains, collect pollen grains from over 20 *Arabidopsis* flowers in order to get a higher density. For the observation of pollen tubes, collect pollen grains from 10 fresh *Arabidopsis* flowers will be enough.
3. To germinate pollen, incubate the medium at 28 °C for 2 h. The pollen tube is suitable for further observation or subject to fixation at about 200 µm (Figure 1D).

C. Actin staining with phalloidin conjugated to fluorescent dye (Video 1)



Video 1. Typical procedure for pollen grain and pollen tube fixation and actin staining

1. Several chemicals were dissolved in solvent according to the product specification and store as stock solution (Recipe 3).
2. To treat pollen cells with LatB, dilute LatB into liquid PGM to certain concentrations, e.g., 150 nM or 100 nM. Considering that actin structures in pollen tube are more sensitive to the application of LatB than that in pollen grains, usually, 100 nM LatB was applied to pollen tube and 150 nM LatB was applied to pollen grains. After incubation with LatB for 30 min, pollen cells were subjected to fixation with MBS. DMSO in PGM was used as the control.
3. Fix the pollen cells with 300 µM MBS for 1 h at 28 °C. Adding 100 µl MBS-PGM for each slice.
4. Remove MBS-PGM and penetrate the pollen cells by 0.05% NP-40, 150 µM MBS in PGM for 10 min.
5. Wash samples with TBS + 0.05% NP40 (Recipe 4) for three times. Keep 100 µl TBS + 0.05% NP40 on samples for 10 min each time.
6. Stain samples with 200 nM Alexa Fluor 488 phalloidin in TBS over night at 4 °C in humid square Petri dish. 30 µl phalloidin buffer is enough for each slice.
7. Wash samples before observation with TBS once.

Notes:

- a. The concentration of DMSO in the PGM should be below 1%.
 - b. Always pay attention to the morphology of the samples. Burst of pollen grain, swell pollen tube tip, and burst of pollen tube, may indicate the inadequacy of buffer.
 - c. Replace new parafilm at Step C6 can avoid buffer slipping from the solid PGM.
 - d. Avoid exposure dye to bright light all the time. Cover square Petri dish in tin foil after adding dye.
- D. Visualization of actin filaments with laser scanning confocal microscope
1. Mount the solid PGM pieces to glass bottom dish to image with Olympus FV1000MPE multiphoton laser scanning confocal microscope equipped with a 100x objective (numerical aperture of 1.4).
 2. Excite samples with 488 nm argon laser and collect emission light at 505-605 nm wavelength. Zoom pollen grain and pollen tube by 2x, image optical slice at 0.5 μm step size.
Note: For image quantification, acquisition parameters must be identical in all images. Avoid overexposure.

Data analysis

To quantify the amount and organization of actin filaments in pollen tubes and pollen grains, the representative results can be found in Figure 2.

1. Import raw images (*i.e.*, oib files for Olympus microscope) into ImageJ software (<http://imagej.nih.gov/ij/>; version 1.52n). Filamentous structures can be distinguished in individual optical slice (Figure 2Aa, Figure 2Ba).
2. Quantify the fluorescent intensity to determine the relative amount of filamentous actin in pollen grains and pollen tubes. Perform the maximum intensity projection of the Z-stack images by ImageJ “Z project” function (Figure 2Ab, Figure 2Bb, Figure 2Cb). Select a 5 μm x 10 μm region at the tip of pollen tube by “Specify” function under “Selection” menu (Figure 2Ab). Select the whole region of pollen grain with “Polygon selection” function (Figure 2Bb, Figure 2Cb). Compared to actin filaments in untreated pollen grains, actin filaments became fragmented and depolymerized under LatB treatment (Figures 2Ca-2Cb). Measure the mean gray value of the selected region from over 20 pollen tubes or over 30 pollen grains to obtain the average fluorescent intensity. The amount of actin filaments can be quantified via measuring the fluorescent intensity, and it is significantly reduced in LatB treated pollen grains (Figure 2D). Statistical analysis was performed by the Student’s *t*-test with R program (<http://www.r-project.org>).
3. Quantify the filament length and angles formed between actin cables and the pollen tube growth axis by “line” tool to analyze the actin structure. Do “maximum intensity projection” with adjacent 3 or 4 slices to facilitate the distinction of individual filaments. Measure angle and length

in slices of the semi-projected images. Plot data and do kernel density estimation with R program (<http://www.r-project.org>) (Figures 2Ac-2Ad).

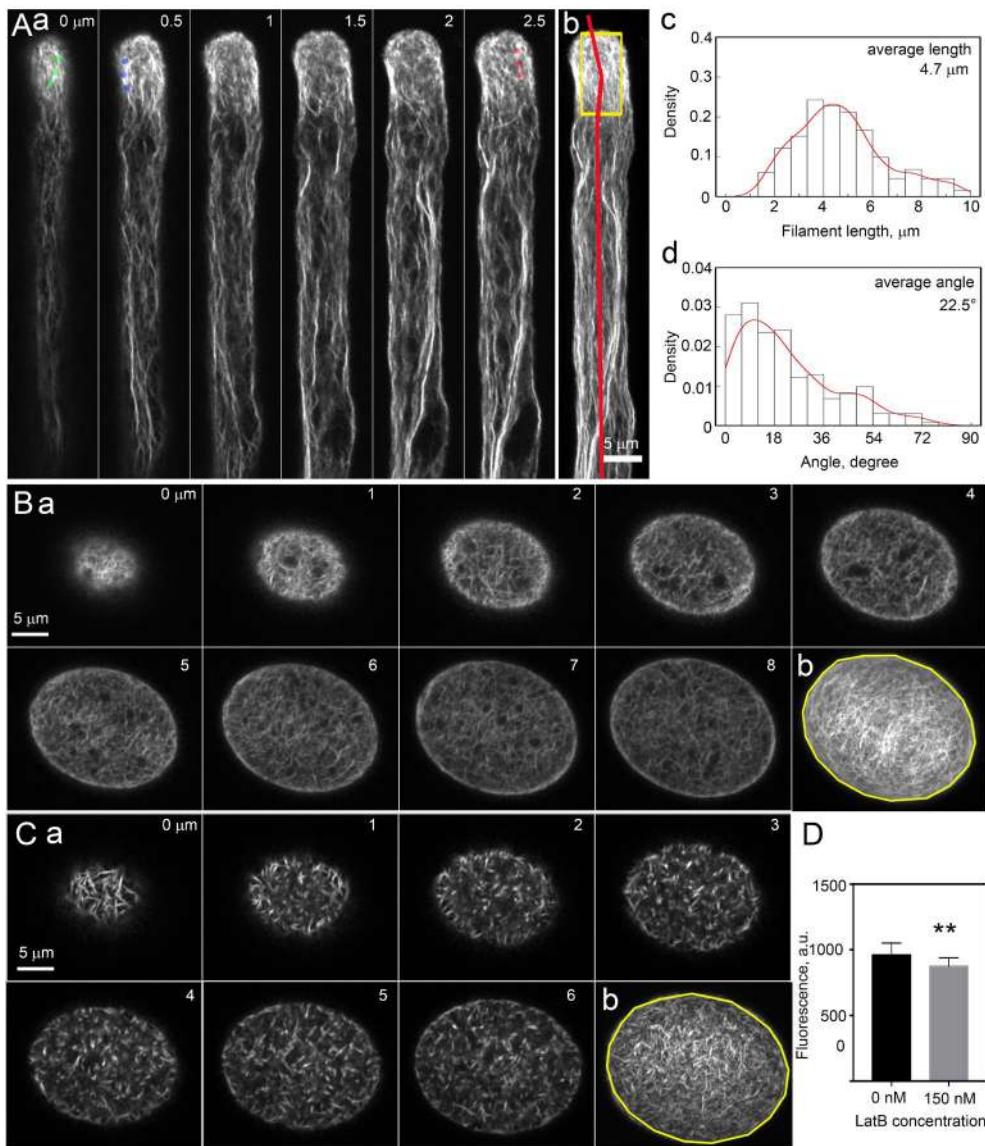


Figure 2. Observation and Quantification of Actin Structures in Pollen Tube and Pollen Grain. A. a), Optical Z-slice images of a pollen tube, with 0.5 μm z-step-size. Individual actin filaments can be distinguished in slices, which were marked by green, blue, and red dots, respectively. A. b), Maximum intensity projected pollen tube shown in (a). Fluorescent intensity can be measured from this kind of images in order to indicate the relative amount of filamentous actin. The yellow box indicates actin filaments at the apex and subapex of the pollen tube. The grow axis of the pollen tube is indicated by red line. Scale bar = 5 μm . A. c), Histogram of the length of actin filaments in yellow boxed region. A. d), Histogram of the angles formed between actin filaments and the growth axis in yellow boxed region. B. a), Optical Z-slice images of a pollen grain, with 1 μm z-step-size. B. b), Maximum intensity projected pollen grain in (a). Fluorescent intensity in yellow circle region will be measured. Scale bar = 5 μm . C. a), Optical

Z-slice images of a pollen grain after being subjected to the treatment with 150 nM LatB. The images were displayed with z-step-size at 1 μm . C. b), Maximum intensity projected pollen grain shown in (a). Fluorescent intensity of phalloidin staining within yellow circle region was measured. Scale bar = 5 μm . D. Quantification of the fluorescent intensity in LatB treated and untreated pollen grains. $n = 30$, ** $P < 0.01$ by Student's *t*-test.

Recipes

1. Individual stocks with Milli-Q water

100 mM CaCl₂

100 mM Ca(NO₃)₂

100 mM MgSO₄

1% H₃BO₃ (wt/vol)

Keep stocks at 4 °C and use within 6 months

2. Pollen germination medium

1 mM CaCl₂

1 mM Ca(NO₃)₂

1 mM MgSO₄

0.01% H₃BO₃ (wt/vol)

18% (wt/vol) sucrose

Adjust pH to 6.85-7.0 with KOH

For solid PGM, add 0.8% agarose

3. Stock solution

100 mM MBS (in DMSO)

5 mM LatB (in DMSO)

6.6 μM Alexa Fluor 488 phalloidin (in methanol)

Aliquote and store them at -20 °C freezer in order to avoid repeated freeze-thaw cycles

4. Tris Buffered Saline (TBS)

50 mM Tris (diluted from 1 M stock, pH 7.4)

200 mM NaCl

400 mM Sucrose

Acknowledgments

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Competing interests

The authors declared that they have no conflicts of interest to this work.

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Sulfatase Assay to Determine Influence of Plants on Microbial Activity in Soil

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[Abstract] Sulfatase activity is often used as a measure of the activity of soil microorganisms. It is thus a suitable tool to investigate the response of microbes to plants. Here we present a method to determine the influence of various *Arabidopsis* genotypes on the function of soil microbiota using the sulfatase as a quantitative measure. We grew the plants in soil/sand mix under control conditions and measured the sulfatase activity in soil using a spectrophotometric determination of the product. This protocol can be used to test the contribution of individual genes to control of microbiome assembly through analysis of mutants as well as the influence of environment on plant-microbe interactions.

Keywords: *Arabidopsis*, Plant-microbe interactions, Soil, Microbiome, Organic sulfur, Sulfatase, Bacteria

[Background] Plants in their natural environment interact with plethora of microorganisms, pathogenic as well as beneficial. Many microorganisms are beneficial to plants, e.g., by improving their immunity or their nutrition (Kertesz and Mirleau, 2004; Jacoby et al., 2017; Stringlis et al., 2018). Indeed, the role of bacteria in plant sulfur nutrition has long been recognized (Kertesz and Mirleau, 2004). Sulfur is present in soil mainly bound to organic compounds and thus not available to plants. However, bacteria and fungi can metabolize such organosulfur compounds and release the sulfate group to the rhizosphere, where it can be utilized by plants, and improve so plant sulfur nutrition (Gahan and Schmalenberger, 2014). One of the enzymes catalyzing such reactions is sulfatase. This enzyme, catalyzing the reaction $X-O-SO_3 + H_2O \rightarrow X-OH + HSO_4^-$, is found in many organisms including bacteria, fungi, and humans, but is not present in plants (Gunal et al., 2019). Sulfatase is induced by sulfate limitation and is the basis of plant growth promoting effects of some bacteria (Kertesz and Mirleau, 2004). Sulfatase activity in soil reflects the activity of microbial communities and can be used for estimation of soil health after various treatments (Tejada et al., 2006; Zaborowska et al., 2018) alongside activities of, e.g., β -glucosidase, cellobiohydrolase, chitinase, leucine aminopeptidase phosphatase, and tyrosine aminopeptidase (Maharjan et al., 2017).

However, the sulfatase can be used also as a tool to study plant-microbe interactions. The role of the plant microbiome in improving plant performance and fitness has been increasingly recognized and great progress in the understanding of the assembly of plant microbiome has been achieved (Bulgarelli et al., 2013; Bai et al., 2015). Clearly, plants shape their microbiome composition, even though the mechanisms are largely unknown. However, most microbiome studies are based on DNA sequencing and therefore taxonomic description of the microbiome composition (Jacoby et al., 2017). We have used

sulfatase activity to identify mechanisms by which plants shape their microbiome (Koprivova *et al.*, 2019). Through analyzing the effects of *Arabidopsis* accessions on sulfatase in soil and using the activity for genome-wide association mapping we revealed an important role of the phytoalexin camalexin in the interactions between plant roots and rhizosphere bacteria (Koprivova *et al.*, 2019). The sulfatase assay, which was adapted from (Margesin *et al.*, 2014), proved to be an excellent tool to assess the microbiome activity and the effect of plant genotype on such activity. Therefore, here we present a protocol not only for the core enzymatic activity but also for a full assessment of the effects of *Arabidopsis* genotypes (accessions or mutants) on microbiome function.

Materials and Reagents

1. Tape
2. 0.5 ml Eppendorf tubes
3. Toothpicks
4. 1.5 ml cuvettes
5. Plastic trays, Plant Pots Direct, Heavyweight full seed tray (no holes), catalog number: 2012137PT (Figure 1)
6. Plastic inserts (Plant Pots Direct, Seed Tray Inserts 40, catalog number: 2012111PT) (Figure 1)
7. Plastic Petri dishes (Sarstedt, catalog number: 821.473)
8. Pipette tips (Sarstedt)
9. 2 ml plastic tubes (Sarstedt, catalog number: 72.695.500)
10. Plastic rack for 2 ml tubes, Eppendorf
11. Plastic cuvettes (Brand, catalog number: 759115)
12. Seeds of *Arabidopsis thaliana* (can be obtained, e.g., from NASC *Arabidopsis* Stock Centre, <http://arabidopsis.info/BasicForm>)
13. Sand, Quarzwerke (Frechen, Germany)
14. Soil (e.g., CAS11 soil, Bulgarelli *et al.*, 2012)
15. Murashige Skoog (MS) medium (Duchefa Biochemie, catalog number: MO222.0025), stored at RT
16. Sucrose (Sigma, catalog number: S7903-1KG), stored at RT
17. Potassium-4-nitrophenyl sulfate (Sigma, catalog number: N3877-1G), stored at -20 °C
18. P-nitrophenol (Merck, catalog number: 48549), stored at -20 °C
19. Calcium chloride dihydrate (Sigma, catalog number C3881-1KG), stored at RT
20. Sodium hydroxide (NaOH) (Sigma, catalog number: 71687-500G), stored at RT
21. Sodium acetate trihydrate (Sigma, catalog number: S8625-500G), stored at RT
22. Calcium nitrate tetrahydrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$) (Merck, catalog number: C1396), stored at RT
23. Potassium nitrate (KNO_3) (Merck catalog number: P8394), stored at RT
24. Potassium dihydrogenphosphate (KH_2PO_4) (Merck, catalog number: 1.04873), stored at RT
25. Ferric EDTA (Fe-EDTA) (Merck, catalogue number: E6760), stored at RT

26. Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) (Merck, catalog number: M2670), stored at RT
27. Liquid nitrogen
28. Glacial acetic acid (Merck, catalog number: A6283), stored at RT
29. Sodium hypochlorite solution ($NaClO$), 12%, Cl (Roth, catalog number: 9062.4)
30. Hydrochloric acid (HCl) 37% (Merck KGaA, catalog number: 1.00317.1000), stored at RT
31. Agarose (Sigma, catalog number: A9539-500G), stored at RT
32. Toluene (Sigma, catalog number: 34866-100 ml), stored under fume hood at RT
33. Modified Long Ashton solution (see Recipes)
34. 0.5 M Acetate buffer (see Recipes)
35. 0.005 M p-nitrophenyl solution (see Recipes)
36. 0.5 M calcium chloride solution (see Recipes)
37. 0.5 M sodium hydroxide solution (see Recipes)
38. Standard p-nitrophenol solution 10 mM (100 ml) (see Recipes)
39. Half strength MS medium with sucrose (1 L) (see Recipes)

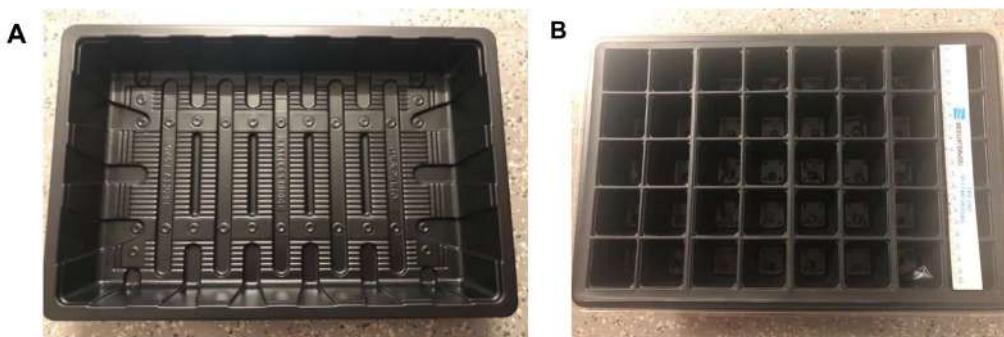


Figure 1. Photos of (A) plastic trays and (B) inserts used for plant growth

Equipment

1. Glass beaker 250 ml
2. Forceps
3. Pipettes (Eppendorf)
4. Balances (Sartorius)
5. Glass beaker
6. Sanyo growth chamber, 10 h light/14 h darkness, 22 °C
7. Rotating shaker, LTF (Labortechnik, Intelli-Mixer, RM-2L)
8. Vortex (LMS, model: VTX-3000L)
9. Incubator for 37 °C (Thermo Scientific, Heratherm Incubator)
10. Centrifuge (Eppendorf, 5424)
11. Spectrophotometer (Eppendorf)
12. Fume hood

13. Computer
14. Desiccator

Procedure

A. *Arabidopsis thaliana* seed sterilization

1. Place small portion of seeds (ca. 10 µl) into 0.5 ml Eppendorf tubes.
2. Place open tubes into tube rack inside of desiccator (Figure 2).
3. Add 125 ml of sodium hypochlorite solution into 250 ml glass beaker and place near the seeds.
4. Add 2.5 ml of concentrated HCl into the liquid, which forms chlorine gas, and quickly close the desiccator lid.
5. Sterilize seeds for 3 h.

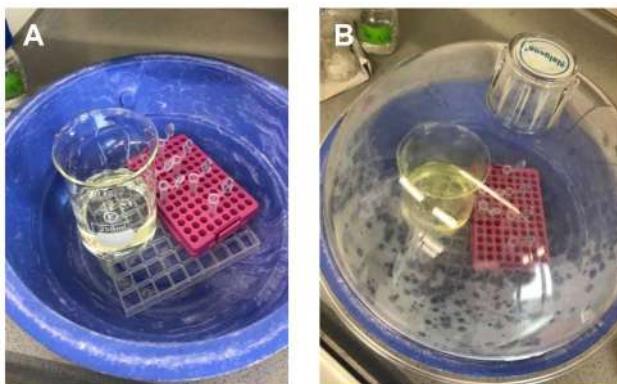


Figure 2. Photos of (A) open or (B) closed desiccator for seed sterilization

B. Initial plates preparation

1. Autoclave half-strength MS medium with 0.8% agarose and 0.5% sucrose and pour it into round Petri dishes, let it set.
2. Carefully place sterile *Arabidopsis* seeds onto agar, using sterile toothpicks, approx. 1 seed per cm².
3. Seal the plates with tape.
4. Place the plates into fridge for 2-3 days for stratification.
5. Place plates into a plant growth cabinet for 9 days (10 h light/14 h dark; 22 °C, 100 µE m⁻² s⁻¹).

C. Preparation of trays

1. Mix soil with sterile (autoclaved) sand 1:9 (V/V).
2. Place plastic insert into the tray.
3. Fill inserts with soil-sand mix.
4. Water slightly
5. Using forceps carefully transfer one seedling per insert, covering roots lightly.

6. Water a bit more, to keep soil moist.
7. Cover tray with the lid.
8. Place trays into plant growth cabinet (10 h light/14 h dark; 22 °C, 100 $\mu\text{E m}^{-2} \text{s}^{-1}$) for 2 weeks, uncover it after 3 days, and water daily with modified Long Ashton solution.

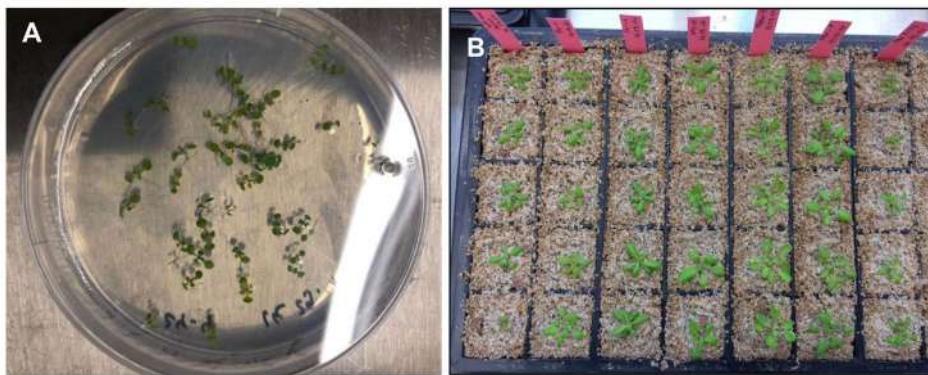


Figure 3. Photos of (A) Petri dish with seedlings before transfer and (B) trays with *Arabidopsis* ecotypes ready for material collection

- D. Collection of samples for sulfatase activity
 1. Carefully remove plants from soil.
 2. Into a 2 ml microcentrifuge tube collect about 1 g of the soil-sand mix, which was closest to the roots of the plant, *i.e.*, rhizosphere, record fresh weight.
 3. Collect at least 2 samples per plant.
 4. Freeze in liquid nitrogen.
- E. Sulfatase activity measurement
 1. Defrost the samples in a rack.
 2. Add 400 μl of 0.5 M acetate buffer.
 3. Vortex each sample for at least 5 s.
 4. Under fume hood add 25 μl of toluene.
 5. Close tubes and vortex for at least 5 s.
 6. Place tubes into rotating rack for 6 min at 100 rpm.
 7. Add 100 μl of p-nitrophenyl sulfate solution under fume hood.
 8. Vortex each sample for 10 s.
 9. Place tubes for additional vigorous shaking for 5 min in an Eppendorf shaker at 1,000 rpm.
 10. Place rack with the tubes into 37 °C incubator for 1 h, mixing the whole rack every 10 min by reversing several times.
 11. To stop the reaction under fume hood add 100 μl of 0.5 M CaCl_2 solution and 400 μl of 0.5 M sodium hydroxide solution.
 12. Vortex each sample for 10 s.
 13. Centrifuge tubes at RT at maximum speed for 20 min.

14. Under fume hood carefully transfer the supernatant into plastic 1.5 ml cuvettes.
15. Measure absorption at 400 nm. Use water as blank.
16. Prepare standards by diluting 0, 20, 40, 80, 120, 160, and 200 μ l of 10 mM p-nitrophenol standard to 1 ml H₂O and measure in the same way.
17. Calculate the p-nitrophenol content of the samples from a calibration curve (standards: 0, 200, 400, 800, 1,200, 1,600, and 2,000 nmol ml⁻¹ p-nitrophenol).
18. Using recorded FW data and incubation time of 1 h calculate sulfatase activity in nmol g FW⁻¹ h⁻¹.

Data analysis

The assay determines the end concentration of the sulfatase product, p-nitrophenol, using a calibration curve (Figure 1) and the activity can be calculated from the weight of soil and time of the assay. All calculations can be easily performed in standard office software, e.g., Excel (Table 1). Five biological replicates with two technical replicates each should be used for each plant genotype.

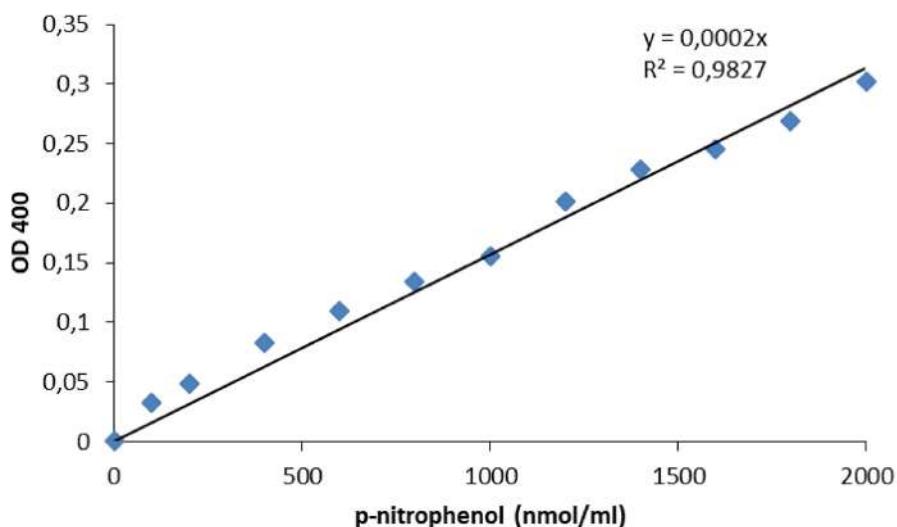


Figure 4. Calibration curve for measurement of sulfatase activity. Standard p-nitrophenol solution was diluted by water to contain 0-2,000 nmol per ml and OD₄₀₀ was measured.

Table 1. Example of calculation of sulfatase activity

sample ID	soil FW ¹ (g)	OD400	p-nitrophenol (nmol)	sulfatase activity (nmol/g FW/h)	Average
Col-0-1	0.1123	0.183	915	8147.8	
Col-0-2	0.1422	0.203	1015	7137.8	
Col-0-3	0.123	0.261	1305	11641.4	
Col-0-4	0.11	0.161	805	6098.5	
Col-0-5	0.111	0.176	880	7926.5	
Col-0-6	0.1121	0.209	1045	9319.5	8409.938
WI-0-1	0.1012	0.1174	587	5800.4	
WI-0-2	0.1211	0.0823	411.5	3398.0	
WI-0-3	0.1113	0.0787	393.5	3535.5	
WI-0-4	0.1241	0.0999	499.5	4025.0	
WI-0-5	0.1117	0.1042	521	4664.3	
WI-0-6	0.1003	0.1092	546	5443.7	4477.805

¹The soil FW is adjusted from the FW of soil/sand sample and the ratio of soil/sand mix, here 10% soil.

Notes

The assay can easily be adapted for different plant species, different sizes of pots, or different soils.

Recipes

1. Modified Long Ashton solution

1.5 mM Ca(NO₃)₂·4H₂O

1 mM KNO₃

0.75 mM KH₂PO₄

0.1 mM Fe-EDTA

0.75 mM MgCl₂·6H₂O

pH 5.7

2. 0.5 M acetate buffer, pH 5.8 (1 L)

64 g sodium acetate trihydrate

1.7 ml glacial acetic acid

Stored at 4 °C

3. 0.005 M p-nitrophenyl sulfate solution (100 ml)

0.1287 g of p-nitrophenyl dissolved in 100 ml of 0.5 M acetate buffer

Stored at 4 °C

4. 0.5 M calcium chloride solution (1 L)

73.5 g CaCl₂·2H₂O dissolved in 1 L H₂O

Stored at RT

5. 0.5 M sodium hydroxide solution (1 L)

20 g NaOH dissolved in 1 L H₂O

Stored at RT

6. Standard p-nitrophenol solution 10 mM (100 ml)

139 mg p-nitrophenol dissolved in 100 ml H₂O

Stored at 4 °C

7. Half strength MS medium with sucrose (1 L)

2.2 g MS medium

5 g sucrose

8 g agarose

pH 5.7 with 1 M KOH

Acknowledgments

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Competing interests

The authors declare no competing interests.

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Insect Feeding Assays with *Spodoptera exigua* on *Arabidopsis thaliana*

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[Abstract] Plant-insect interaction is an important field for studying plant immunity. The beet armyworm, *Spodoptera exigua*, is one of the best-known agricultural pest insects and is usually used to study plant interactions with chewing insects. Here, we describe a protocol for insect feeding assays with *Spodoptera exigua* larvae using model host plant *Arabidopsis thaliana*, which is simple and easy to conduct, and can be used to evaluate the effect of host genes on insect growth and thus to study plant resistance to chewing insects.

Keywords: Plant-insect interaction, chewing insects, *Spodoptera exigua*, *Arabidopsis thaliana*, Insect feeding assay

[Background] Plants face a variety of biotic stresses throughout their lives, such as herbivore attack and pathogen infection. The beet armyworm, *Spodoptera exigua*, is a worldwide phytophagous pest with a broad host range, damaging various vegetable crops and causing considerable economic agricultural losses (Howe and Jander, 2008; Hu et al., 2013). *Spodoptera exigua* larvae usually feed on both foliage and fruit, and are extremely destructive. *Arabidopsis thaliana* is a host plant of *Spodoptera exigua*, and is also a classic model plant to study plant resistance to herbivores. Here, we describe a method adapted from our previous work (You et al., 2019) to conduct the feeding assays with *Spodoptera exigua* larvae on *Arabidopsis thaliana* rosette leaves. By counting the weight of the larvae after feeding leaves from different genotypes, we were able to evaluate plant resistance to herbivore attacks in the laboratory settings.

Materials and Reagents

1. Square Petri dishes (100 mm × 100 mm, Beijing Ruiaizhengte Biological Technology Co., Ltd., catalog number: YC-HC99050)
2. Petri dishes (150 mm, Corning, catalog number: 430599)
3. 1.5 ml microtubes (AXYGEN, catalog number: MCT-150-C)
4. Pipet tips (AXYGEN, catalog number: T-200-Y and T-1000-B)
5. Micropore tape (3M, Micropore™, catalog number: 1530C-0)

6. Parafilm (Bemis, catalog number: PM-996)
7. Toothpick (Suncha, catalog number: YQ1250)
8. Black cloth (Beiyang, catalog number: 13000133)
9. *Arabidopsis thaliana*
10. *Spodoptera exigua* (KEYUN)
(<https://item.taobao.com/item.htm?spm=a1z09.2.0.0.10672e8dnIXuwi&id=567208183626&u=pmk8luh1123>)
11. Artificial diet (Ingredients: wheat germ, yeast, carrageenan, konjac powder, sorbic acid, vitamin C, corn oil, and linoleic acid; KEYUN)
(<https://item.taobao.com/item.htm?spm=a1z09.2.0.0.10672e8dnIXuwi&id=43498077050&u=pmk8luh74e5>)
12. Murashige & Skoog basal medium with vitamins (Phyto Technology Laboratories, catalog number: M519)
13. Bacto agar (BD, Bacto™, catalog number: 214010)
14. 10% Bleach (KAO, 600 ml)
15. 1 M KOH solution (Aladdin, catalog number: P112281)
16. Sucrose (Sinopharm Chemical Reagent Co., Ltd., catalog number: 10021418)
17. Diethyl ether (Sinopharm Chemical Reagent Co., Ltd., catalog number: 10009328)
18. Sterile distilled water
19. Nutritional soil (moss peat:vermiculite = 2:1, PINDSTRUP, type: 0-10 mm)
20. ½ MS medium (see Recipes)
21. 0.8% agar medium (see Recipes)

Equipment

1. Pipettes (Gilson, Pipetman® G)
2. Graduated cylinder
3. Reagent bottle
4. Refrigerator or a cold room
5. Tweezers
6. Plant growth chamber
7. Square pot
8. Autoclave
9. Laminar flow hood
10. Balance
11. Canon camera
12. Ruler

Software

1. Microsoft Excel

Procedure

A. Cultivation of *Arabidopsis thaliana* plants

1. Prepare the $\frac{1}{2}$ MS solid medium, and pour enough media into square Petri dishes (100 mm × 100 mm) to cover approximately half of the depth of the dish (40 ml).
2. Surface-sterilize *Arabidopsis* seeds in 1.5 ml microtubes by soaking in 10% bleach for 15 min, then remove all bleach residue by rinsing five times with sterile distilled water.
3. Sow the seeds in the square Petri dishes containing $\frac{1}{2}$ MS medium (Figure 1A), and seal the dishes with Micropore tape.
4. Place the dishes in the dark at 4 °C for 2 days to allow for efficient and synchronous germination.
5. Transfer the dishes to the growth chamber set at 22 °C with a 10-h light/14-h dark photoperiod for 10 days.
6. Transplant the seedlings into square pots filled with nutritional soil (Figure 1B), and keep them in the growth chamber (22 °C, 10-h light/14-h dark) for another 4 weeks (Figure 1C).

Note: Be careful not to damage the roots of the seedlings. Take care of the plants to prevent them from being affected by herbivores or pathogens.

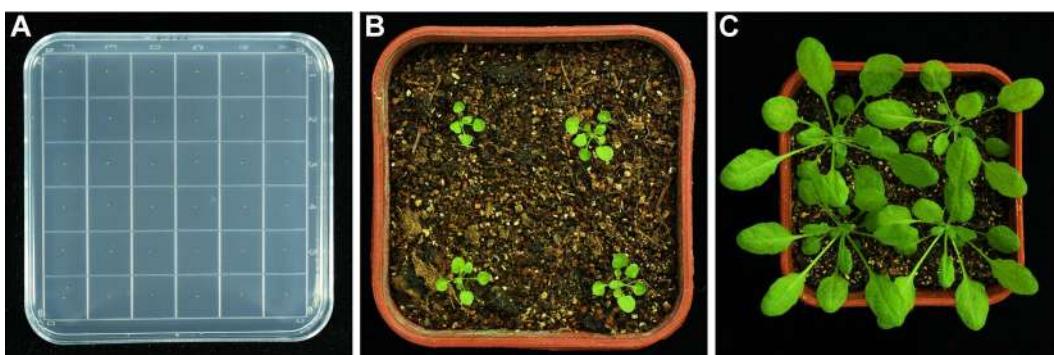


Figure 1. The cultivation of *Arabidopsis* plants. A. Sterilized *Arabidopsis* seeds were sowed on the square dish containing $\frac{1}{2}$ MS medium. B. Ten-day old *Arabidopsis* seedlings were transplanted into a pot filled with nutritional soil. C. *Arabidopsis* seedlings grown in pot for 4 weeks are used for insect feeding assays.

B. Preparation of *Spodoptera exigua* larvae

1. Place the *Spodoptera exigua* eggs in a square Petri dish, and seal the dish with Micropore tape (Figure 2A).
2. Place the dish in the 27 °C incubator with relative humidity of 40-50% for hatching (about 2-3 days).

- As soon as the eggs hatch, add artificial diet to the dish (Figure 2B) and continue incubation for 5 days.

Note: Cut the artificial diet into small pieces of 1-1.5 cm square and placed them in the Petri dish at a certain interval from the eggs or larvae. Usually, after 5 days of feeding, the larvae become third-instar larvae.

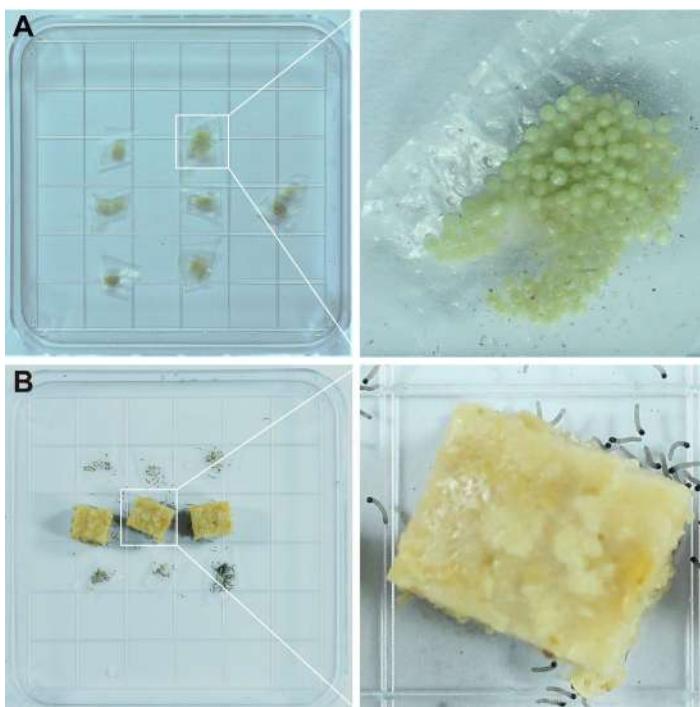


Figure 2. The hatching of *Spodoptera exigua* eggs. A. *Spodoptera exigua* eggs. B. Newly hatched larvae and the artificial diet.

- Use a toothpick to transfer the larvae into a new Petri dish (Video 1 and Figure 3A) and starve them for 12 h before being used in the experiments (Figure 3B).

Note: This step needs to be careful and gentle. Usually, we use a water-soaked toothpick to let the larvae catch and gently transfer the larvae to prevent them from being injured.

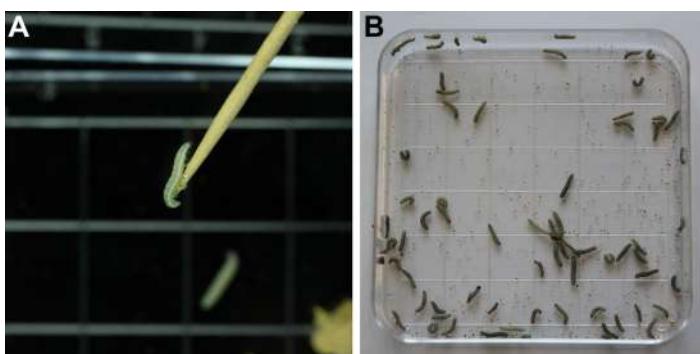
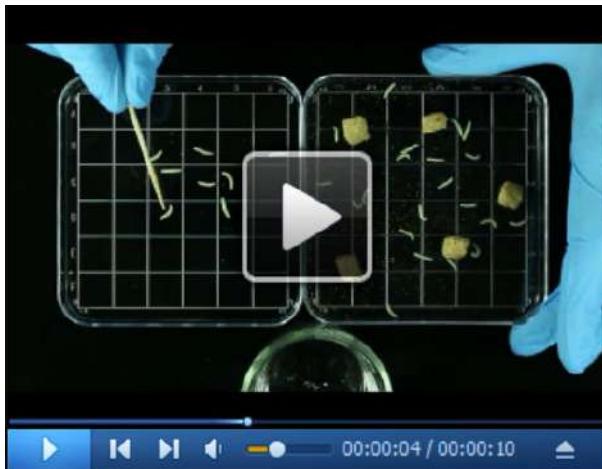


Figure 3. The starvation treatment of *Spodoptera exigua* larvae. A. A larva clutching a

toothpick. B. Third-instar larvae starved for 12 h.



Video 1. How to catch a larva with a toothpick?

C. Insect feeding assays

1. Prepare and pour enough 0.8% agar into plastic Petri dishes (150 mm) to cover approximately half of the depth of the dish.

2. Cut mature rosette leaves of similar size from *Arabidopsis* plants grown in soil for 4 weeks, and place them in a plastic Petri dish (150 mm) containing 0.8% agar (Figure 4A).

Note: If the leaves of different genotypes are similar in shape and size, we usually arrange them in the way shown in Figure 4A. And if the shape and size of the leaves vary greatly, it is required to weigh the leaves each time to ensure that the same weight of leaves are added.

3. Use a water-soaked toothpick to gently transfer 15 starved third-instar larvae into the Petri dish containing rosette leaves.

Note: This step needs to be careful and gentle.

4. Seal the Petri dishes with Micropore tape, and put them in the growth chamber (22 °C, 10-h light/14-h dark).

5. Feed the larvae for 3 days (Figure 4B), and replace the leaves in each Petri dish by fresh leaves every day.

Note: Add fresh leaves in time to ensure that the larvae have enough food. The feeding days can be adjusted according to the experimental conditions.

*As the plant hormone jasmonate (JA) plays a vital role in regulating plant defense response against herbivore attack, we conducted the insect feeding assays using the wild type (WT) plants and the coi1-2 mutant, which harbors a point mutation of the JA receptor gene CORONATINE INSENSITIVE1 (COI1) (Xu et al., 2002), and compared their resistance to the *S. exigua* larvae (Figures 4C and 4D).*

D. Collection of experimental results

1. After three days of feeding, transfer the larvae into a new Petri dish, and weigh all *Spodoptera*

exigua larvae from each Petri dish.

2. Place a cotton ball soaked in ether in the Petri dish, and seal the dish with parafilm.
 3. When the larvae are unconscious, use a small pair of tweezers to gently place them on a black cloth, arrange them from large to small, and take photos (Figure 4C).
- Note: Steps D1 and D3 should be gentle to prevent the larvae from being injured and spit out green oral secretions.*

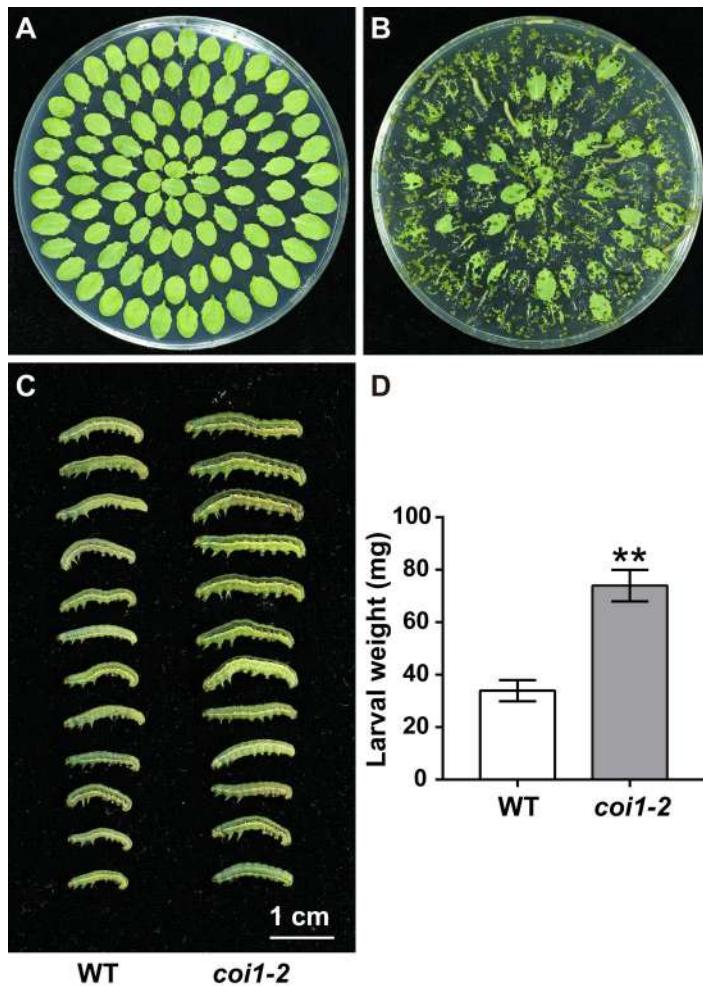


Figure 4. Insect feeding assays with *Spodoptera exigua* larvae. A. Mature rosette leaves were cut and placed in the plastic Petri dish (150 mm) containing 0.8% agar. B. The leaves after feeding by *Spodoptera exigua* larvae for 1 day. C. *Spodoptera exigua* larvae after feeding on rosette leaves of WT and *coi1-2* plants for 3 days. Scale Bar = 1 cm. D. Average weight of the larvae fed with rosette leaves of WT and *coi1-2* plants for 3 days. Data represent means \pm SD ($n = 3$). Asterisks indicate significant differences from the WT according to Student's *t*-test at **, $P < 0.01$.

Data analysis

Count the weight of each larva in each dish, and calculate the average. Statistical analysis should be done by calculating the average of three independent experiments and standard deviation using Microsoft Excel or any other statistical analysis software. Significance of the difference between two samples can be obtained by performing the Student's *t*-test (Figure 4D). Usually, the larvae grow well after eating the leaves. In some cases, one or two larvae will die, and avoid using the dead larvae for analysis. Otherwise, collect all larvae for data analysis.

Recipes

1. ½ MS medium
 - 2.215 g Murashige & Skoog basal medium with vitamins
 - 10 g sucrose
 - 8 g Bacto agar
 - Add ddH₂O to 1,000 ml
 - Use KOH to adjust pH to 5.8
 - Autoclave at 15 psi, 121 °C for 15 min
2. 0.8% agar medium
 - 8 g Agar
 - Add ddH₂O to 1,000 ml
 - Autoclave at 121 °C for 15 min

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Competing interests

The authors declare that they have no competing interests.

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Single-run HPLC Quantification of Plant Cell Wall Monosaccharides

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[Abstract] The plant cell wall is a complex network of polysaccharides and proteins that provides strength and structural integrity to plant cells, as well as playing a vital role in growth, development, and defense response. Cell wall polysaccharides can be broadly grouped into three categories: cellulose, pectins, and hemicelluloses. Dynamic interactions between polysaccharides and cell wall-associated proteins contribute to regions of flexibility and rigidity within the cell wall, allowing for remodeling when necessary during growth, environmental adaptation, or stress response activation. These polysaccharide interactions are vital to plant growth, however they also contribute to the level of difficulty encountered when attempting to analyze cell wall structure and composition. In the past, lengthy protocols to quantify cell wall monosaccharides contributing to cellulose as well as neutral and acidic cell wall polysaccharides have been used. Recently, a streamlined approach for monosaccharide quantification was described. This protocol combines a simplified hydrolysis method followed by several runs of high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Here, we present an updated version of this protocol in which we can analyze all nine cell wall monosaccharides in a single high-performance liquid chromatography HPAEC-PAD gradient profile. The inclusion of an enzymatic starch degradation, as well as alternate internal standards for added quantification accuracy, and a ready-to-use Python script facilitating data analysis adds a broadened scope of utility to this protocol. This protocol was used to analyze *Arabidopsis* light-grown seedlings and dark-grown hypocotyls, but is suitable for any plant tissues.

Keywords: Cell wall, Cellulose, HPLC, Monosaccharides, Saeman hydrolysis, *Arabidopsis*

[Background] Understanding plant cell wall structure and composition have been at the forefront of both academic and industrial plant research for many years. Polysaccharide and cell wall protein interactions provide structure to plant cells, while also actively playing important biological roles during growth and adaptation to diverse external conditions (Cosgrove, 2005 and 2016; Kesten et al., 2017). The plant cell wall acts as an important physical barrier during biotic or abiotic interactions, but can also provide an endogenous source of signaling molecules released during stress response (Cosgrove, 2005 and 2016; Kesten et al., 2017 and 2019). These cell wall-derived molecules can activate signaling cascades to alert the plant host of an invading pathogen or a need to redirect growth resources. Cell wall polysaccharides are also important for industrial uses—pectins are widely used in the food and cosmetics industries, while cellulose is important for the food, paper, and textile industries (Pettolino

et al., 2012). As such, efficient and informative methods for characterizing plant cell wall polysaccharides are vital in both academia and industry to further understand the biological role of the plant cell wall and improve ease of polymer extraction while reducing waste during industrial applications.

Experiments from the 1940s conducted in wood established the use of dilute acid hydrolysis at high temperature to study sugar decomposition into monomers (Saeman, 1945). Such studies have provided a foundation for many current protocols, including the one presented here. Streamlining this analysis has allowed us to improve both the accuracy and efficiency of our measurements. Other recently described protocols have established the combination of the widely-used trifluoroacetic acid (TFA) hydrolysis approach with a single HPLC run, or a dual sulfuric acid-based hydrolysis method, referred to as a “one-step two-step sulfuric acid hydrolysis” approach, with multiple HPAEC-PAD runs (Zhang *et al.*, 2012; Voiniciuc *et al.*, 2016; Yeats *et al.*, 2016a and 2016b). This “one-step two-step” approach makes use of a Saeman hydrolysis procedure followed by what has been previously referred to as a “matrix hydrolysis” (Yeats *et al.*, 2016a). Saeman hydrolysis, the “two-step” portion of this approach, is essentially the addition of concentrated sulfuric acid hydrolysis (72% sulfuric acid) directly to plant material in order to swell the sample. After one hour at room temperature, water is added to the sample to dilute the sulfuric acid concentration to 4%. This portion of the hydrolysis is then performed at 121 °C for one hour. The use of a dilute acid hydrolysis immediately following a strong acid swelling hydrolysis allows the release of glucose from all possible sources, including heavily cross-linked and rigidly packed cell wall components, such as crystalline cellulose. The “one-step” portion of the hydrolysis, also called the “matrix hydrolysis”, refers to the hydrolysis of samples only at 121 °C with 4% sulfuric acid. By simply treating samples with a “matrix hydrolysis”, only monosaccharides from easily hydrolysable sources will be released. Therefore, the difference between these two hydrolyses allows the quantification of crystalline cellulose in what is referred to as a “one-step two-step” hydrolysis approach. In our protocol, we combine the efficient “one-step two-step” sulfuric acid hydrolysis described by Yeats *et al.* (2016a) with the inclusion of an optional enzymatic starch degradation and the use of alternate internal standards to elute all cell wall sugars and quantify glucose derived from crystalline cellulose in a single HPAEC-PAD run. Our additions to the previously mentioned protocols contribute significant improvements in both ease and accuracy of monosaccharide measurement that will greatly benefit the field of plant cell wall research.

Materials and Reagents

1. 2 ml screw-cap tubes (Sarstedt, catalog number: 72.694.006)
2. 50 ml conical tubes (Greiner Bio-One, catalog number: 7.210 261)
3. 500 µl autosampler Snap Ring vials (Sigma, catalog number: 27422)
4. Autosampler vial lids (Sigma, catalog number: 24757)
5. Stainless steel 25 ml grinding jars (Retsch, catalog number: 02.462.0119)
6. Stainless steel 12 mm grinding balls (Retsch, catalog number: 05.368.0032)
7. Tin weigh boats, 5 x 9 mm (Santis Analytical AG, catalog number: SA76981103)

8. 0.22 µm sterile PES-membrane filter (Life Systems Design AG, catalog number: 99255)
9. Metal spatula
10. Plastic weighing papers (HuberLab, catalog number: 12.9702.080)
11. Conical tubes (Grenier Bio, catalog number: 7.210 261)
12. Nylon mesh, 60 µm pore size (Sefar Nitex, catalog number: 3A03-0060-110-00)
13. Square Petri plates (Greiner Bio-One, catalog number: 7.688.102)
14. Homogenizing beads (depending on amount of material):
 - a. Small glass beads (2.85-3.45 mm beads, Roth, catalog number: A557.1)
 - b. Large metal balls (12 mm beads, Retsch, catalog number: 05.368.0032)
15. CarboPac PA20 column (3 x 150 mm, Thermo Fisher Scientific, catalog number: 060142)
16. CarboPac PA20 guard column (3 x 30 mm, Thermo Fisher Scientific, catalog number: 060144)
17. *Arabidopsis thaliana*
18. Ethanol (HCl Shop, ETH Zurich, catalog number: 02000107)
19. Chloroform (Sigma, catalog number: 25693)
20. Methanol (HCl Shop, ETH Zurich, catalog number: 02000342)
21. Acetone (Sigma, catalog number: 24201-4X2.5L-GL-R)
22. (Optional) Enzymatic starch degradation
 - a. Amyloglucosidase (Sigma, catalog number: 10102857001)
 - b. α-Amylase (Sigma, catalog number: 10102814001)
23. Sulfuric acid (Sigma, catalog number: 258105)
24. Ultrapure water (Milli-Q or equivalent)
25. Monosaccharide analysis standards:
 - a. L-fucose (Sigma, catalog number: F2252-5G)
 - b. D-glucose (Sigma, catalog number: G7528-1KG)
 - c. D-galactose (Sigma, catalog number: 48260)
 - d. D-xylose (Sigma, catalog number: W360600-SAMPLE)
 - e. D-mannose (Sigma, catalog number: 63579)
 - f. L-arabinose (Roth, catalog number: 5118.2)
 - g. L-rhamnose (Sigma, catalog number: W373011-SAMPLE-K)
 - h. D-galacturonic acid monohydrate (Sigma, catalog number: 48280)
 - i. D-glucuronic acid (Sigma, catalog number: G5269-10G)
26. (Optional) Alternate internal standards
 - a. D-sedoheptulose (CarboSynth, catalog number: MS139006)
 - b. D-ribose (Sigma, catalog number: R7500-5G)
27. Sodium hydroxide, 50% solution in water (Sigma, catalog number: 415413)
28. Sodium acetate, anhydrous (Sigma, catalog number: 32319-1KG-R)
29. Lugol solution (Sigma, catalog number: 32922-6X1L)
30. Liquid nitrogen

Equipment

1. Freeze-dryer (Christ, Alpha 2-4)
2. Microcentrifuge (Eppendorf, model: 5424 R)
3. Sample concentrator (Stuart, model: SBHCONC/1)
4. Autoclave-compatible rack (Karter Scientific, catalog number: 125A7)
5. Microbalance (Mettler Toledo MX5)
6. Tissue homogenizer (Retsch MM200)
7. Micro-centrifuge tube shaker (Eppendorf ThermoMixer F1.5)
8. Tube rotator (Labinco LD79, catalog number: 79000)
9. Speed-vacuum centrifuge (Eppendorf Concentrator Plus)
10. Autoclave (Thermo Fisher Scientific, Sterico, Varioklav)
11. Heating block (Stuart, model: SBH130D)
12. Autosampler (Dionex, model: AS-1)
13. Dionex ICS-5000 (Dionex, model: DC-5)
 - a. ED Electrochemical Detector (without cell, product number: 072042)
 - b. ED Cell (no reference or working electrode, product number: 072044)
 - c. Gold (Au) on Polytetrafluororethylene (PTFE) Disposable Electrode (product number: 066480)

Software

1. Chromeleon 8 (Thermo Fisher Scientific)
Available for a fee at <https://www.thermofisher.com/order/catalog/product/CHROMELEON7>
2. Microsoft Excel
Available for a fee at <https://www.office.com>
3. GraphPad Prism
Available for a fee at <https://www.graphpad.com>
4. Spyder5 (Anaconda3)
Freely available at <https://www.anaconda.com>
5. Python 3.6
Freely available at <https://www.python.org>

Procedure

- A. Generating and preparing plant material
 1. Grow plants in desired conditions

Note: Plant samples can be collected from any desired growth method; we suggest the following *in vitro* method for *Arabidopsis* material generated from either light-grown (any day cycle) seedlings or dark-grown hypocotyls.

- a. Gas or liquid sterilize seeds and stratify at 4 °C for 2-3 days.
 - b. Plates: prepare ½ MS + 1% sucrose + 0.9% agar square Petri plates; sterilize nylon mesh and lay on top of agar plates; sow approximately 100-120 seeds at top of plates; collect material at desired time point in 50 ml conical tubes; remove seeds before processing.
 - c. For light-grown plants, grow plants in light for 14 days under long-day conditions (16 h light, 8 h dark) with light optimized for *Arabidopsis* (130-150 $\mu\text{E m}^{-2} \text{s}^{-1}$) at 20-22 °C.
 - d. For dark-grown hypocotyls, sow multiple lines of seed per plate (without nylon); leave seeds sown on plates in light for 2-4 h; cover in several layers of aluminum foil and allow to grow in climate-controlled chamber for 5 days at 20-22 °C.
2. Leave plants in the dark for 24-48 h prior to harvesting to deplete starch reserves; if not possible, an enzymatic starch degradation can be performed after homogenization of plant material (proceed to Step A5).
 3. If analyzing aerial plant tissues (containing chlorophyll), harvest material in enough 70% ethanol to fully submerge plant material, and continue exchanging ethanol until chlorophyll is depleted and the liquid no longer has any trace of green colour.
If non-chlorophyll-containing plant portions are to be analyzed, harvest and flash freeze samples with liquid nitrogen and proceed to grinding in Step A5.
 4. Remove ethanol and use freeze-dryer to dry material over 2 days (or longer if necessary).
 5. Grind all plant material using tissue homogenizer with 12 mm diameter metal balls and metal containers.

Notes:

- a. If generating only a small amount of material, it is possible to harvest roots into microcentrifuge tubes and use a tissue homogenizer with racks for microcentrifuge tubes and glass beads for grinding. In this case, aliquot ≤ 200 mg plant material to ensure thorough homogenization.
- b. When collecting plant material, it is imperative to avoid collection of agar, soil, or any growth media containing sugars with samples (N.B. agar will contribute to galactose quantification). Nylon mesh can be used as described above to prevent agar adhesion to roots.

- B. Starch degradation conducted as previously described (Hostettler *et al.*, 2011) with modifications as follows:
1. Aliquot ground plant material up to ~0.5 ml in a 2 ml micro-centrifuge tube.
 2. Add 1 ml 80% ethanol (v/v) and heat samples at 95 °C for 10 min. Mix well by vortexing each sample for 10-15 s.
 3. Centrifuge at room temperature (RT) at 3,000 $\times g$ for 5 min; discard supernatant.

Note: All centrifugation steps should be performed at RT unless otherwise indicated.

4. Continue with the following washing steps: shaking in a ThermoMixer for 10 min during each wash, centrifuging at 3,000 $\times g$ for 5 min, vortexing to re-suspend pellet between washes, discard supernatant, and add the next washing solution: 1 ml 50% (v/v) ethanol, 1 ml 20% (v/v) ethanol, 1 ml water, and finally 1 ml 80% (v/v) ethanol.

Note: After Step B4, the final wash should be mostly clear, but pellet may still be green.

5. Dry the pellet at room temperature, using a speed-vacuum centrifuge, or in an oven at 60 °C for at least 30 min or over-night until completely dry. Re-suspend in 400 μl water and vortex to mix.
6. Boil at 95 °C for 10-15 min—do not cool on ice.

7. Prepare a digestion mixture of 9 parts amyloglucosidase and 1 part α -amylase.

Note: Calculate volume of digestion mixture needed based on total number of samples, keeping in mind that 20 μl of the mixture is required per sample. One sample requires 18 μl of amyloglucosidase and 2 μl α -amylase.

8. Add 380 μl 0.22 M sodium acetate to 20 μl of the prepared 9:1 amyloglucosidase: α -amylase mixture; combine with 400 μl sample.

Note: If processing larger sample volumes, sample can be resuspended in larger volume of water. For digestion, simply combine equal parts sample with digestion mixture and proceed as indicated.

9. Digest at 37 °C for a minimum of 2 h.

10. Check if there is remaining starch by staining a small portion of the plant material with Lugol solution.

Note: Lugol staining suggested procedure:

- a. Mix 20 μl sample with 80 μl 100% ethanol.
- b. Boil for 5 min at 90 °C.
- c. Centrifuge for 5 min at 5,000 $\times g$. Discard supernatant.
- d. Add 25 μl Lugol solution; check for colour change after 5 min.
 - i. Lugol solution can be used at full concentration without dilution of purchased solution.
 - ii. Colour change to a deep blue/black colour indicates presence of starch.
 - iii. If colour change is observed, continue digestion overnight and check with Lugol again. Continue digestion until colour change is no longer observed (Figure 1).
 - iv. Once no colour change is observed, proceed to Step A11.

11. Centrifuge samples at 5,000 $\times g$ for 5 min and proceed with the insoluble fraction remaining.

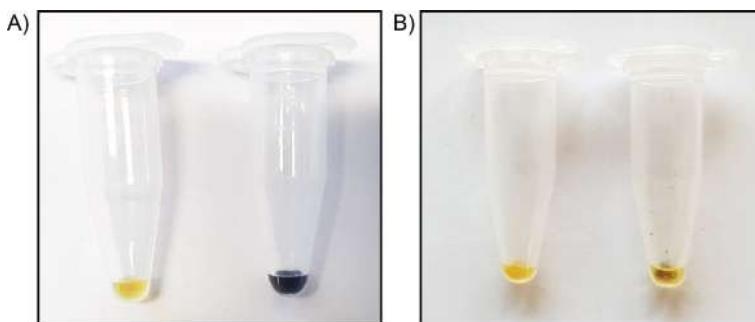


Figure 1. Lugol stain reveals presence or absence of starch. A. Full concentration Lugol alone has a faint yellow colour (left tube), while Lugol added directly to starch extracted from corn shows a strong dark brown/purple colour change (right tube). B. Both tubes contain cell wall AIR material; the tube on the left has been subjected to starch degradation, while the tube on the right has not. Following the procedure described in Step A10, addition of 25 μ l full concentration Lugol will stain AIR samples containing starch a dark brown/purple colour, as observed in the right tube. If the Lugol remains yellow when added to the sample, starch is not present, and one may proceed with Step A11.

- C. Cleaning and production of final cell wall-derived alcohol insoluble residue (AIR) preparation
 1. Aliquot de-starched insoluble fraction up to 0.5 ml in a 2 ml micro-centrifuge tube.
 2. Add 1.5 ml of a 1:1 methanol:chloroform (v/v) mixture to sample and vortex to mix thoroughly. Mix samples for 2 h using the tube rotator set to 15 rpm, or any standard tube mixer.
 3. Centrifuge at 10,000 $\times g$ for 5-10 min at room temperature and remove supernatant.
 4. Add 1.5 ml RT acetone and vortex to mix thoroughly. Mix for 30 min using a tube rotator set to 15 rpm or any standard tube mixer.
 5. Centrifuge at 10,000 $\times g$ for 5-10 min at room temperature and remove supernatant.
 6. Dry the final pellet at room temperature, using a speed-vacuum centrifuge, or in an oven at 60 °C for at least 30 min or overnight until completely dry. The final product is the cell wall-derived alcohol insoluble residue (AIR).
- D. Sample and standard hydrolysis conducted as described in Yeats *et al.* (2016a) (see Figure 2 in this manuscript). Briefly, the method is summarized as follows:

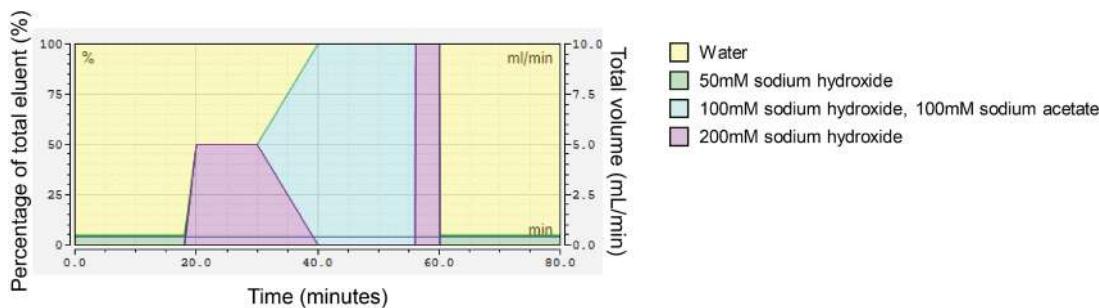


Figure 2. Gradient profile of single HPLC run to elute all cell wall monosaccharides. Each block of colour corresponds to a particular eluent, indicated in the legend on the right. The primary y-axis (left) features percentage, referring to what percentage each eluent comprises the final mixed eluent that flows through the column, with the total always equaling to 100%. The secondary y-axis (right) indicates the volume (ml/minute) used up per eluent, relating to the percentage of the total mixed eluent to which each individual eluent contributes. The x-axis indicates the amount of time (minutes) for which the indicated eluent composition should proceed.

1. Weigh 1 ± 0.1 mg AIR per technical replicate into 2 ml screw-cap tubes using a microbalance and tin weigh boats; record final weight (required for analysis steps).

Notes:

- If spatula used for weighing AIR needs to be cleaned in between samples, do not use any kind of tissue/paper towel. Use the plastic weighing papers to wipe and rinse thoroughly with 100% ethanol in between samples.
- We recommend a minimum of two technical replicates per sample per hydrolysis (meaning, 2 technical replicates for samples subjected only to matrix hydrolysis, and 2 technical replicates subjected to Saeman hydrolysis + matrix hydrolysis) and three biological replicates for each experimental analysis.
- Prior to sample hydrolysis, internal standards (sedoheptulose or ribose) can be added directly to weighed AIR aliquots. Allow internal standards to completely dry for 30 min or longer as necessary, either at room temperature or using a sample concentrator.
 - For our assays, we added 150 μ g of sedoheptulose to AIR material; however, this amount must be optimized based on the sample dilution that will be measured considering the working range of the instrument of choice.
 - Addition of the standards (ribose or sedoheptulose) is not mandatory. However, they provide an added measure of certainty to ensure consistency of hydrolysis and quantification.
 - Sedoheptulose worked better for this analysis as we observed a "background" peak with the same retention time as ribose. However, with a slightly different instrument or sample, ribose may be used. This should be tested prior to hydrolysis of all samples and standards.

2. One sample set will be subjected to Saeman as well as matrix hydrolysis, while a second sample set will be subjected only to matrix hydrolysis.

Note: Previously, this method was described using autoclaving as the hydrolysis method (Yeats et al., 2016a). We confirm this as an efficient method of hydrolyzing upwards of 50 samples at once. However, we also confirmed the validity of the HPLC analysis and quantification if hydrolysis is accomplished using a heating block at 121 °C for 1 h. However, data is more consistent when using autoclaving to accomplish hydrolysis (Figure 3), as is demonstrated by reduced error. Further, it appears hydrolysis of galacturonic acid may be incomplete using heating block hydrolysis, although all other monosaccharide values seem to be consistent across either method. It may also be possible to use a heating block at a lower temperature (80–100 °C) for a longer hydrolysis time, however this should be tested and optimized.

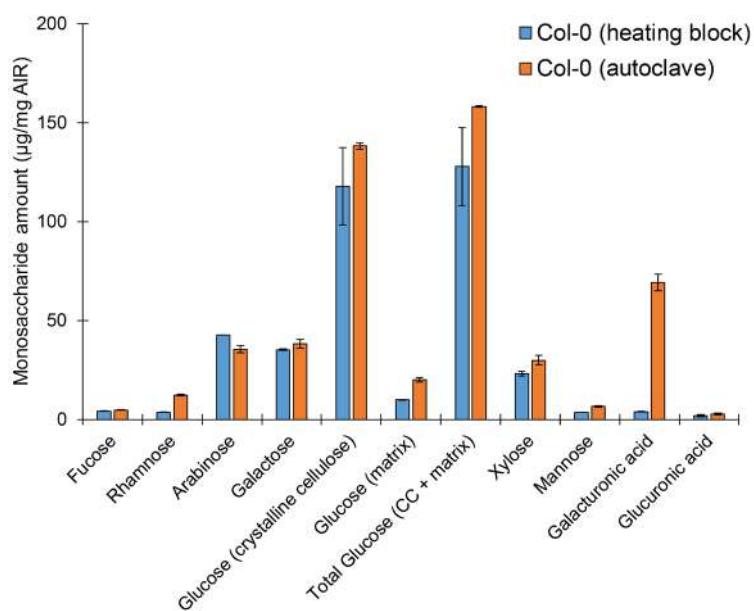


Figure 3. Sample hydrolysis may be accomplished using either a heating block or autoclave, with minor differences. Monosaccharide elution profile of cell wall AIR derived from light-grown wild-type Col-0 seedlings. A minimum of two technical replicates per sample per hydrolysis method were used. Bars represent average of 2 biological replicates ± standard error.

3. For standard curve analysis, make a 100 µg stock solution containing all monosaccharides for quantification as well as the appropriate internal standard. Dilute into appropriate standard concentrations based on assaying range.

Note: Recommended concentrations to generate the standard curve are as follows: 0.05 µg, 0.1 µg, 0.5 µg, 1 µg, 2 µg, 5 µg.

4. To consider sugar-specific losses during hydrolysis and calculate monosaccharide-specific correction factors, prepare two recovery standards by combining 500 µl of the 100 µg standard mixture with 900 µl water.

- a. One recovery standard is subjected to the same conditions as the matrix hydrolysis samples (water + acid, hydrolysis for 1 h at 121 °C).
- b. The second recovery standard is treated with acid, but is not subjected to heat hydrolysis.
5. After hydrolyses are complete, allow samples to cool at room temperature and centrifuge for 1 min at 20,000 x g to pellet any insoluble material. The supernatant is used in the next step.

E. HPLC analysis

1. Dilute sample supernatants as required before injection (1:10, 1:20, 1:50, or 1:100 dilutions may be used depending on the starting material and detector sensitivity) and pipet into autosampler vials.

Notes:

- a. *Appropriate standards and dilutions may vary based on samples or detector sensitivity; it is recommended to test standards as well as sample dilutions thoroughly to optimize conditions before completing processing of all material.*
- b. *It is strongly recommended to randomize order of sample analysis and run a standard after every 10-15 samples to ensure sensitivity and accuracy of measurement is consistent.*
2. Make eluents; purge with and maintain under helium gas, or as directed by manufacturer (Rohrer, 2017). Any eluents containing sodium acetate must be filtered using a 0.22 µm PES filter. All eluents must be purged for a minimum of 10 min before addition of 50% sodium hydroxide solution, followed by additional purging of a minimum of 10 min after addition.
Eluent A = water
Eluent B = 50 mM sodium hydroxide
Eluent C = 100 mM sodium hydroxide, 100 mM sodium acetate
Eluent D = 200 mM sodium hydroxide
3. Inject 10 µl of each standard, recovery standard, and sample onto a 3 x 150 mm CarboPac PA20 column equipped with a 3 x 50 mm CarboPac PA20 guard column.
4. Maintain column temperature at 36 °C with a flow rate of 0.4 ml/min.
5. Use the following elution profile to elute all monosaccharides and standards (Figure 4, Table 1): 0-18 min 4.8% B, 95.2% A; 18-20 min linear gradient to next condition; 20-30 min 50% D, 50% A; 30-40 min linear gradient next condition; 40-56 min 100% C; 56-56.1 min linear gradient to 50% D; 56.1-60 min 50% D; 60-60.1 min change to next condition; 60.1-80 min 4.8% B, 95.2% A to equilibrate column back to starting conditions:

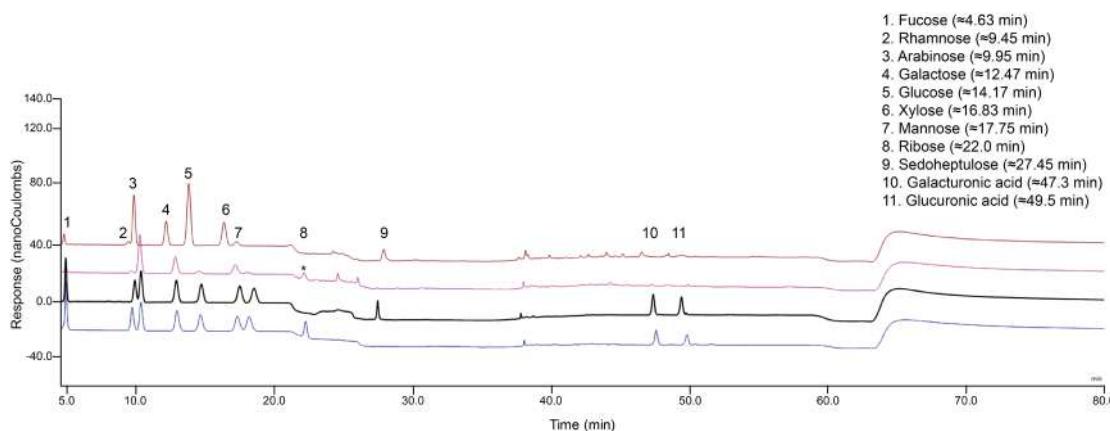


Figure 4. Elution profile of cell wall monosaccharides in a single HPAEC-PAD run. Four separate injections are presented: 1 µg/ml standards mixture using ribose as an internal standard (blue), 1 µg/ml standards mixture using sedoheptulose as an internal standard (black), and an example of a wild type Col-0 matrix hydrolysis profile (hydrolyzed using a heating block) (pink), and an example of a wild type Col-0 Saeman hydrolysis + matrix hydrolysis profile with sedoheptulose added as an internal standard (hydrolyzed using a heating block) (brown). After sample hydrolysis (pink), a background peak (*) appeared that clearly overlaps with the ribose peak (blue).

Notes:

- a. *Retention times of peaks may shift as more samples run, therefore regular “column flushing” and monitoring of column performance may be necessary.*
- b. *A short “column flushing” period is incorporated into the gradient profile (~5 min of 100 mM NaOH at 56 min); however, it is also possible to run a flushing profile periodically as follows: 30 min 100% Eluent C followed by 30 min 100% Eluent D with a short equilibration step (approximately 10-15 min) back to starting conditions (95.2% Eluent A, 4.8% Eluent B).*
- c. *If significant changes in column performance are observed, immediate action to fully flush the column as per the manufacturer’s instructions must be taken. In short, we accomplished column flushing by conducting the following:*
 - i. *Disconnect column from detector and general machine flow by turning the electrode/detector off and unscrewing the column outlet.*
 - ii. *Wash column with 2 M NaOH (allowing flow-through to drip into a waste receptacle) for 1 h; adjust flow rate until pressure reaches similar level to running pressure during sample analysis (in this case, ~2,200 psi).*
 - iii. *Re-equilibrate column with starting conditions (in the case of this protocol, 4.8% Eluent B, 95.2% Eluent A) for 30 min; again, adjust flow rate until pressure reaches similar level to running pressure during sample analysis, and collect flow-through in a waste receptacle.*
 - iv. *Re-attach column to the system, and run a water sample followed by a standard before continuing with sample analysis using the normal gradient profile.*

- d. Technical notes from the manufacturer were consulted thoroughly for this analysis (Basumallick and Rohrer, 2017).

Table 1. Plant cell wall monosaccharide elution gradient steps

Time (min)	Eluent A (%)	Eluent B (%)	Eluent C (%)	Eluent D (%)
0-18	95.2	4.8	0	0
20	50	0	0	50
30	50	0	0	50
40	0	0	100	0
56	0	0	100	0
56.1	0	0	0	100
60	0	0	0	100
60.1	95.2	4.8	0	0
80	95.2	4.8	0	0

Data analysis

1. All standard curve and sample peaks were integrated using Chromeleon 8.0 software and analyzed using Microsoft Excel as described in Yeats *et al.* (2016a). GraphPad Prism was used for statistical analyses and generating graphs.
2. In order to facilitate data entry into Microsoft Excel, a customized peak-calling analysis method in Chromeleon and a Python script can be used to copy integrated curve values into a sorted, transposed Excel spreadsheet. Values can subsequently be copied into a similar analysis sheet as the one described in Yeats *et al.* (2016a).
 - a. Raw data from Chromeleon runs must be saved as Excel spreadsheets within the same folder as the [Python script](#).
 - b. Peak calling can be adjusted directly in Chromeleon to limit output to only sugars desired for analysis.
 - c. The Python script can be modified to analyze all cell wall sugars, as well as glucosamine if desired, or other additional sugars.

Note: If using the Python script for analysis, adjust the number of inputs in Line 9 of the script to reflect the number of peaks that will be called by the Chromeleon software based on the test parameters chosen in the software.

Conclusions:

This protocol can be used to efficiently quantify all cell wall monosaccharides using a single HPAEC-PAD gradient profile. This quantification will allow the reliable characterization of monosaccharide composition, and allows for the determination of proportions of glucose that come from both the crystalline and non-crystalline portions of the plant cell wall. For example, using wildtype (Col-0),

and the well-characterized cellulose-deficient mutant and *prc1-1*, we demonstrate that this method is sufficient for resolving differences between cell wall mutants (Figure 5). Additionally, this analysis has already been used in a recent study to quantify differences in glucose derived from crystalline cellulose (Kesten et al., 2019). This method is simple, reliable, and consistent, and can be used to better understand cell wall monosaccharide compositional changes in a biological context. Past methods rely on multiple hydrolyses approaches or HPLC gradients to quantify neutral cell wall monosaccharides, uronic acids, and cellulose separately. Therefore, this analysis represents a streamlined alternative to total cell wall monosaccharide analysis.

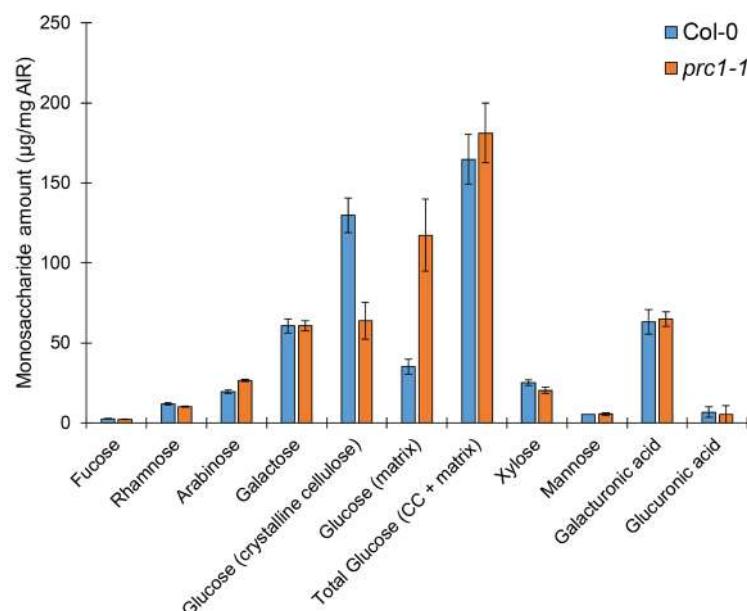


Figure 5. Cell wall mutants can be clearly distinguished from wild-type using this hydrolysis and analysis method. Monosaccharide elution profile of cell wall AIR derived from dark-grown Col-0 or cellulose-deficient *prc1-1* hypocotyls. A minimum of two technical replicates per sample per hydrolysis method were used. Bars represent average of 2 or 3 biological replicates (for Col-0 or *prc1-1*, respectively) ± standard error.

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Competing interests

There are no conflicts of interest or competing interest.

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MAMP-triggered Medium Alkalization of Plant Cell Cultures

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[Abstract] Plants recognize a wide variety of microbial molecules to detect and respond to potential invaders. Recognition of Microbe-Associated Molecular Patterns (MAMPs) by cell surface receptors initiate a cascade of biochemical responses that include, among others, ion fluxes across the plasma membrane. A consequence of such event is a decrease in the concentration of extracellular H⁺ ions, which can be experimentally detected in plant cell suspensions as a shift in the pH of the medium. Thus, similarly to reactive oxygen species (ROS) accumulation, phosphorylation of MAP kinases and induction of defense-related genes, MAMP-induced medium alkalization can be used as a proxy for the activation of plant immune responses. Here, we describe a detailed protocol for the measurement of medium alkalization of tobacco BY-2 cell suspensions upon treatment with two different MAMPs: chitohexamers derived from fungal cell walls (NAG6; N-acetylglucosamine) and the flagellin epitope flg22, found in the bacterial flagellum. This method provides a reliable and fast platform to access MAMP-Triggered Immunity (MTI) in tobacco cell suspensions and can be easily adapted to other plant species as well as to other MAMPs.

Keywords: Plant immunity, Host-microbe interactions, Elicitor, Effector Biology, Phytopathology

[Background] Throughout evolution, plants developed the ability to detect microbe-derived molecules and mount immune responses that seize detrimental interactions (Boutrot and Zipfel, 2017). The microbial-associated molecular patterns (MAMPs) that induce such immune responses are often broadly conserved structural components of microbes, such as chitin from fungi and flagellin from bacteria (Cook et al., 2015). Recognition of MAMPs by extracellular plant receptors leads to MAMP-triggered immunity (MTI), which contributes to halt microbial invaders and maintain plant health (Böhm et al., 2014).

At the molecular level, MAMP perception is followed by an orchestrated set of biochemical events. Firstly, within seconds to few minutes, influx of Ca²⁺ and H⁺ ions occurs, leading to membrane depolarization and extracellular alkalization (Ranf et al., 2011). Subsequently, production of reactive oxygen species (ROS) is observed, which may locally block pathogen growth and mediate downstream

signaling (Bigeard *et al.*, 2015). Phosphorylation of MAP kinases occurs within minutes after MAMP perception and promotes transcriptional activation of defense-related genes, many of which encode antimicrobial proteins or enzymes involved in the synthesis of hormones and secondary metabolites (Bigeard *et al.*, 2015). Lastly, accumulation of antimicrobial compounds and callose deposition are observed, further hampering microbial growth and spread.

Mechanistic understanding of the plant immune system and its activity in plant-microbe interactions has advanced through methods that monitor many of the biochemical events underlying MTI. The most widely used experimental approaches are based on detection of ROS burst, MAP kinase activation, induction of defense-related marker genes and medium alkalinization of cell suspensions (Flury *et al.*, 2013; Bisceglia *et al.*, 2015; Liu *et al.*, 2018). ROS measurement is typically performed by means of luminol-based chemiluminescence assays. MAP kinase activation is detected by western blots using specific antibodies. Transcriptional reprogramming is usually assessed by monitoring the expression levels of one or a few marker genes using real-time PCR. Medium alkalinization assays are based on recording the pH changes in MAMP-treated plant cell suspensions over time using a pH sensor for small volumes. Compared to the other methods, medium alkalinization assays are less laborious and require low cost laboratory equipment. Both model plant species and crops have been studied using medium alkalinization assays (Nühse *et al.*, 2000; Moroz *et al.*, 2017).

Materials and Reagents

1. 12-well cell culture plate (Greiner Bio-One, CELLSTAR®, catalog number: 665180)
2. Serological pipette without tip 10 ml (SARSTEDT, catalog number: 86.1688.010)
3. P200 pipette tips
4. 20 ml syringe (VWR, catalog number: 613-2046)
5. 0.22 µm syringe filter (GE Healthcare, Whatman™ Uniflo, catalog number: 9915-2502)
6. Hexa-N-acetylchitohexaose (two manufacturers are recommended: Santa Cruz Biotechnology, catalog number: sc-222018; Isosep, catalog number: 56/11-0010). Store at -20 °C
Note: Chitoheptaose (NAG7) and chitoctaose (NAG8) or even chitin (Sigma, catalog number: C7170) may be used instead of chitohexaose.
7. Flagellin 22 peptide (flg22: QRLSTGSRINSAKDDAAGLQIA) (Genscript, catalog number: RP19986). Store at -20 °C
8. Gamborg's vitamin Solution 1,000x (sterile) (Sigma-Aldrich, catalog number: G1019-50ML). Store at 4 °C
Note: Before using, inspect for the presence of precipitates. If present, warm the solution up to 42 °C and vortex until all precipitates dissolve.
9. Murashige and Skoog Basal salt mixture (PhytoTechnology Laboratories, catalog number: M524). Store at 4 °C
10. Sucrose (Sigma-Aldrich, catalog number: S5391)
11. Myo-inositol (Sigma-Aldrich, catalog number: I7508)

12. Thiamine hydrochloride (Sigma-Aldrich, catalog number: T1270). Store at 4 °C
13. KH₂PO₄ (Sigma-Aldrich, catalog number: P5655)
14. Tryptone (Oxoid, catalog number: LP0042)
15. 2,4-Dichlorophenoxyacetic acid (Sigma-Aldrich, catalog number: D7299)
16. 2-(N-Morpholino)ethanosulfonic acid (MES) hydrate (Sigma-Aldrich, catalog number: M2933)
17. Kinetin (Sigma-Aldrich, catalog number: K0753)
18. Chitohexamers (NAG6) solution (see Recipes)
19. Flg22 solution (see Recipes)
20. Kinetin stock solution (1,000x) (see Recipes)
21. 2,4-Dichlorophenoxyacetic acid (2,4-D) stock solution (1,000x) (see Recipes)
22. KH₂PO₄ stock solution (see Recipes)
23. Thiamine/myo-inositol stock solution (see Recipes)
24. Tryptone stock solution (10%) (see Recipes)
25. MES/Phosphate-buffered culture medium (see Recipes)
26. Subculturing medium (see Recipes)

Equipment

1. 125 ml Erlenmeyer flasks (Pyrex™, catalog number: 15685767)
2. 250 ml Erlenmeyer flasks (Pyrex™, catalog number: 11902619)
3. P200 adjustable pipette
4. Flow hood (Thermo Scientific, model: 1300 Series Class II, catalog number: 1323TS)
5. Autoclave (VWR, catalog number: 481-0666)
6. pH meter (Mettler Toledo, Seven Compact S220 Basic, catalog number: 30019028). Alternative models may be used as long as the pH resolution is at least 0.001
7. pH sensor InLab Micro for small volumes (Mettler Toledo, catalog number: 51343160)
8. Orbital shaker (LaboTech, model: RS150)

Software

1. Microsoft Excel
2. EasyDirect™ pH Software (Mettler Toledo, 30323214)

Procedure

A. Cell suspension preparation and maintenance

The protocol described here uses *Nicotiana tabacum* cultivar Bright Yellow 2 (BY-2) cell suspensions, which are established from *in vitro*-induced calli (Figure 1A). The BY-2 cell line was produced from tobacco seedlings by Kato *et al.* in 1972 (Kato *et al.*, 1972) and has since been propagated and

shared among plant scientists (Nagata et al., 1992). BY-2 cells are currently used as models in a number of experimental systems and are available from various research groups.

1. To generate a suspension of plant cells, transfer approximately 1 ml of calli to a sterile 125 ml Erlenmeyer flask containing 20 ml of MES/Phosphate-buffered culture medium (Recipe 8) using a sterile spatula or inoculation loop (in the flow hood). Be gentle to avoid unnecessary mechanical stress to the calli. In order to prevent contamination, work carefully and orderly at the flow hood. Incubate at 27 °C in the dark and under gentle agitation (100-120 rpm) in an orbital shaker. The calli will release small cell aggregates that will eventually result in a cell suspension.
2. After 10-14 days, transfer 5 ml of the resulting cell suspension using a 10 ml tipless serological pipette to a sterile 250 ml Erlenmeyer flask containing 45 ml of subculturing medium (Recipe 9). Incubate at 27 °C in the dark and under gentle agitation (100-120 rpm) in an orbital shaker.
3. After 7 days, repeat the subculturing as described in Step A2. At this point, the newly established cell suspension should be similar to the one shown in Figures 1B-1C. Inspect an aliquot of the suspension under the microscope to ensure that it is free from contaminants.
4. For regular maintenance of BY-2 cell suspensions, subculture the cells weekly as described in Step A2. It is advisable to routinely keep two or three independent flasks of cell suspensions to prevent culture loss due to contamination.



Figure 1. *Nicotiana tabacum* cv. Bright Yellow 2 (BY-2) cells growing as calli and cell suspensions. A. Calli growing on solid MES/Phosphate-buffered culture medium. B. Cell suspension just after subculturing. C. A 7-day-old cell suspension. Note the pale-yellow color indicating saturation.

B. MAMP treatment and measurement of medium alkalization

Once established cell suspensions are available (as described in the previous section), MAMP-triggered medium alkalization can be accessed. Aliquots of cell suspension are individually treated with MAMPs and probed for response (Figure 2).

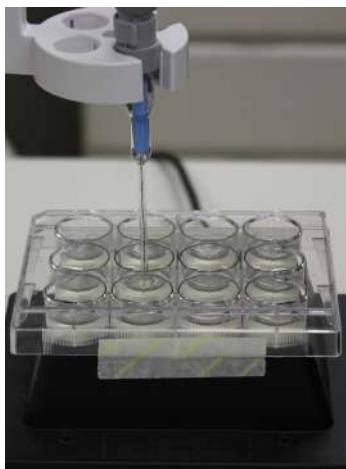


Figure 2. Experimental setup for medium alkalinization measurement. A 12-well microplate containing 2.5 ml of cell suspensions in each well is kept under gentle agitation on an orbital shaker. The pH sensor is inserted so that the probe is completely submerged and does not touch the walls.

1. Carefully transfer a 3- to 5-day-old cell suspension to a 12-well plate (2.5 ml per well) using a 10 ml tipless serological pipette. The cell cultures to be used should be similar to the ones shown in Figure 1C, with a pale-yellow coloration. Dark yellow or brownish colored suspensions are typically stressed and should not be used.
Note: One key factor that affects the medium alkalinization assay is the age of the cell suspension. In our experiments with BY-2 cells, we used 3- to 5-day-old suspensions. However, the optimum age may vary from lab to lab and therefore needs to be determined experimentally.
2. Incubate the plate for at least 2 h and up to 4 h at room temperature and under gentle agitation in an orbital shaker (typically 100-200 rpm; the speed should be just enough to keep the cells in suspension). This step allows the cells to recover from stresses caused by the transference to the plate.
3. Before starting the measurements, adjust the pH meter settings appropriately: measurement resolution of 0.001, endpoint format set as manual and timed interval readings of 3 s. Calibrate the sensor according to the manufacturer's recommendations using calibration standard solutions.
4. Open the EasyDirect™ pH Software on a computer connected to the pH meter and set the export option to Excel. An Excel sheet will automatically be opened.
5. Keeping the plate under agitation on the orbital shaker (100-200 rpm), insert the sensor in one well, carefully adjusting the position to ensure that the sensor is sufficiently inserted (colored tip submerged) and is not touching the walls or the bottom of the well.
6. Press the Start button on the pH meter. The instrument will start recording the pH values in the Excel sheet.
7. Once the measurements become stable (same value for at least 12 s), add 25 µl of 1 µM NAG6 (Recipe 1) (final concentration: 10 nM) or 25 µl of 10 µM flg22 (Recipe 2) (final concentration:

100 nM) to the well. To avoid artefactual changes in pH, equilibrate the elicitors to room temperature for 10 min before adding to the cell suspension. Make sure that each plate includes at least one well treated with elicitor solvent (H_2O) as non-elicited control.

8. Record the pH values for 10 min for NAG6 and 60 min for flg22.
9. After the measurement is complete, remove the sensor from the well, wash thoroughly with ultrapure water and dry using tissue paper.
10. Repeat Steps B5-B9 until all samples included in the experiment have been tested.

Data analysis

The EasyDirect™ pH Software features an export tool that records pH measurements directly into Excel sheets. Therefore, once all the raw data from an experiment is collected, the variation in pH (ΔpH) over time for each tested condition can be calculated by subtracting the pH value measured at time 0 (i.e., the pH of the medium before MAMP treatment) from the pH at each subsequent time point (i.e., the pH of the medium after MAMP treatment). Line plots depicting the variation in pH over time (Figure 3A) or bar plots showing the maximum ΔpH value (Figure 3B) for each treatment may be plotted to visualize the results. Standard deviation should be presented to indicate variation among replicates. It is desirable to have at least 3 biological replicates for each condition tested.

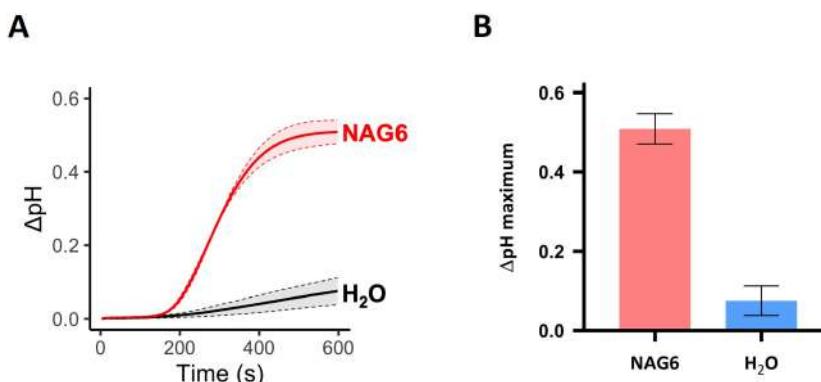


Figure 3. NAG6-induced medium alkalization of tobacco BY-2 cell suspensions. The pH of cell suspensions treated with 10 nM NAG6 or ultrapure water (mock) was recorded for 10 min after the treatment. A. Variation in pH (ΔpH) over time (in seconds) shown in a line plot. B. Maximum variation in pH plotted as a bar graph. Shades in the line graph and error bars in the bar plot indicate standard deviation of three biological replicates.

Notes

1. As an alternative to the tipless serological pipette, it is possible to cut the end of a regular serological pipette or even from a P5000 tip and use it for handling of the cell suspensions.

Recipes

1. Chitohexamers (NAG6) solution
Dissolve in sterile ultrapure water to 1 μ M (1.24 μ g/ml)
Store at -20 °C
2. Flg22 solution
Dissolve in sterile ultrapure water to 10 μ M (22.7 μ g/ml)
Store at -20 °C
3. Kinetin stock solution (1,000x)
 - a. Dissolve 0.1 mg/ml kinetin in 1/10 volume (100 μ l per ml) of 1 M NaOH aqueous solution
 - b. Once the powder is dissolved, complete the final volume with ultrapure water
 - c. Filter sterilize and store at -20 °C
4. 2,4-Dichlorophenoxyacetic acid (2,4-D) stock solution (1,000x)
Dissolve 1 mg/ml 2,4-D in absolute ethanol
Store at -20 °C
5. KH_2PO_4 stock solution
 - a. Dissolve 60 mg/ml KH_2PO_4 in deionized water
 - b. Prepare 10 ml aliquots and store at -20 °C
6. Thiamine/myo-inositol stock solution
 - a. Dissolve 0.1 mg/ml thiamine hydrochloride and 10 mg/ml myo-inositol in deionized water
 - b. Prepare 3 ml aliquots and store at -20 °C
7. Tryptone stock solution (10%)
 - a. Dissolve 10 g/L tryptone in deionized water
 - b. Sterilize by autoclavation or filtration through a 0.22 μ m syringe filter
 - c. Store at room temperature
8. MES/Phosphate-buffered culture medium

Composition:

0.5 g/L 2-(N-Morpholino)ethanesulfonic acid (MES) hydrate

30 g/L sucrose

1x Murashige and Skoog basal salts

100 mg/L myo-inositol

1 mg/L Thiamine hydrochloride (vitamin B1)

0.18 g/L KH_2PO_4

0.22 mg/L (0.1 μ M) 2,4-Dichlorophenoxyacetic acid

1% tryptone

pH 5.7

Preparation (1 L):

- a. Dissolve 4.3 g of Murashige and Skoog basal salt mixture, 30 g sucrose and 0.5 g MES hydrate in 900 ml of deionized water
 - b. Adjust the pH to 5.7 using a 1 M KOH aqueous solution
 - c. Add 3 ml of KH₂PO₄ stock solution, 10 ml of Thiamine/myo-inositol stock solution and 220 µl of 2,4-D stock solution
 - d. Complete the final volume to 1 L with deionized water
 - e. Transfer 18 ml aliquots to 125 ml Erlenmeyer flasks
 - f. Autoclave at 121 °C and 15 psi for 20 min
 - g. After equilibration at room temperature, add 2 ml of tryptone stock solution. The medium is ready to be used
9. Subculturing medium

Composition:

1x Murashige and Skoog basal salts
30 g/L sucrose
1x Gamborg's vitamins
1 mg/L (4.5 µM) 2,4-Dichlorophenoxyacetic acid
0.1 mg/L (0.46 µM) kinetin
pH 5.7

Preparation (1 L):

- a. Dissolve 4.3 g of Murashige and Skoog basal salt mixture and 30 g sucrose in 900 ml of deionized water
- b. Adjust the pH to 5.7 using a 1 M KOH aqueous solution
- c. Complete the final volume with deionized water
- d. Transfer 45 ml aliquots to 250 ml Erlenmeyer flasks
- e. Autoclave at 121 °C and 15 psi for 20 min
- f. After equilibration at room temperature, transfer the autoclaved flasks to a flow hood and add 45 µl of 1,000x Gamborg's vitamin solution (sterile), 45 µl of 2,4-Dichlorophenoxyacetic acid stock solution and 45 µl of kinetin stock solution. The medium is ready to be used

Acknowledgments

This protocol describes in detail the procedure to access MAMP-triggered medium alkalization of cell suspensions used in the research report by Fiorin *et al.* (2018). This research was supported by the São Paulo Research Foundation (FAPESP) through fellowships 09/51018-1, 09/50119-9, 11/23315-1, 13/09878-9 and 14/06181-0.

Competing interests

The authors declare no conflict of interest.

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Purification of Protein-complexes from the *Cyanobacterium Synechocystis* sp. PCC 6803 Using FLAG-affinity Chromatography

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[Abstract] Exploring the structure and function of protein complexes requires their isolation in the native state—a task that is made challenging when studying labile and/or low abundant complexes. The difficulties in preparing membrane-protein complexes are especially notorious. The cyanobacterium *Synechocystis* sp. PCC 6803 is a widely used model organism for the physiology of oxygenic phototrophs, and the biogenesis of membrane-bound photosynthetic complexes has traditionally been studied using this cyanobacterium. In a typical approach, the protein complexes are purified with a combination of His-affinity chromatography and a size-based fractionation method such as gradient ultracentrifugation and/or native electrophoresis. However, His-affinity purification harbors prominent contaminants and the levels of many proteins are too low for a feasible multi-step purification. Here, we have developed a purification method for the isolation of 3x FLAG-tagged proteins from the membrane and soluble fractions of *Synechocystis*. Soluble proteins or solubilized thylakoids are subjected to a single affinity purification step that utilizes the highly specific binding of FLAG-affinity resin. After an intensive wash, the captured proteins are released from the resin under native conditions using an excess of synthetic 3x FLAG peptide. The protocol allows fast isolation of low abundant protein complexes with a superb purity.

Keywords: Protein purification, Membrane protein complexes, *Synechocystis* 6803, Photosystems, FLAG-tag, Affinity chromatography

[Background] Cyanobacteria have been used as the preferred model systems to study the biogenesis and function of photosynthetic protein complexes for decades. The photosynthetic apparatus in cyanobacteria is very similar to eukaryotic systems (algae and plants), but cyanobacteria have all the advantages of prokaryotic models, such as fast growth and small genomes that allow easy genetic manipulation and the use of standard tools of bacterial genetics. Particularly, *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) is established as a favorite model organism in this field, mainly because it is transformable with high efficiency and integrates DNA by functional homologous recombination. Moreover, a glucose tolerant strain of *Synechocystis*, which can proliferate using carbohydrate supplement, enables studying the function of photosynthetic genes by reverse genetics (Williams, 1988).

The biogenesis of photosystem II (PSII), a large membrane embedded pigment-protein complex responsible for photosynthetic water oxidation, has been studied intensively in *Synechocystis* (Nixon et al., 2010). The assembly of the PSII complex from individual subunits requires complicated machinery

of many auxiliary (assembly) factors, which are typically involved in a certain assembly step (Komenda et al., 2012). A conventional approach for the analysis of PSII assembly complexes is using *Synechocystis* mutants lacking one or several PSII subunits. PSII biogenesis is then blocked at a particular step where the missing subunit would bind to an intermediate complex of the assembly process. In such mutants, the otherwise transient PSII pre-complexes can accumulate to a level that allows their detection and even purification, which has traditionally been achieved via His-tagged PSII (core) subunits (Dobáková et al., 2007 and 2009; Boehm et al., 2011 and 2012).

His-tag has several advantages: it is small enough to be easily introduced via a simple primer design, and it is relatively inexpensive to use. However, after His-affinity purification the preparations from *Synechocystis* often contain prominent contaminating proteins (Boehm et al., 2011 and 2012; Liu et al., 2011); therefore, additional purification steps are usually required to obtain the satisfactory quality for functional and structural studies. Additional purification steps may, however, compromise the yield and nativity of the complex of interest. Moreover, the levels of many protein complexes in the cell are simply too low for feasible multi-step purification protocols, PSII assembly intermediates are such an example. For these reasons, a fast, gentle, single-step purification method is highly desirable.

Affinity tags, based on biomolecular interactions offer superior specificity as compared to the general metal affinity of His-tag (Lichty et al., 2005). Large, proteinaceous tags such as small ubiquitin-like modifier (SUMO) or maltose binding protein (MBP) are well suited for the isolation of small soluble proteins due to their stable nature, which can even promote the solubility of the protein (Esposito and Chatterjee, 2006). However, for studying larger and potentially labile membrane-protein complexes, a less bulky tag with minimal interference on protein-protein interactions is preferable. Short peptide tags with high-affinity to proteins or antibodies seem to be the best suited for this purpose. One such tag is the short octapeptide called FLAG (DYKDDDDK; Hopp et al., 1988). Compared to His-, and several other tags, using FLAG-tag results in superior purity with little compromise in yield (Lichty et al., 2005). Moreover, because the elution of FLAG-tagged proteins is possible using a synthetic FLAG-peptide, the usage of disrupting chemicals, high ion concentrations or extreme pH can be avoided. Even though the price per mg purified protein of FLAG-affinity resin is approximately 50 times higher than that of Ni-NTA resin (Lichty et al., 2005); for the purification of labile and/or low-abundant complexes it can well worth the time and costs saved compared to optimizing multi-step purification procedures. Recently, FLAG-tag based approaches have been employed to purify pathogenic variants of the human Huntingtin protein from mammalian and insect cell lines (Harding et al., 2009), as well as the integral membrane Vo-part of the *Pichia pastoris* V-type ATPase (Li et al., 2017).

The FLAG-affinity purification protocol presented here is designed for the purification of proteins tagged with 3x FLAG epitope (DYKDDDDKDYKDDDDKDYKDDDDK). The protocol has been successfully used to resolve the roles of the PSII assembly factors Ycf39 (Knoppová et al., 2014), CyanoP (Knoppová et al., 2016), Pam68 (Bučinská et al., 2018) and RubA (Kiss et al., 2019); as well as pigment-protein complexes containing the chlorophyll-synthase (Chidgey et al., 2014), protoporphyrinogen oxidase (Skotnicová et al., 2018) and ferrochelatase enzymes (Pazderník et al., 2019). Although the method was originally developed for membrane-bound protein complexes, with

minor modifications it can also be applied to soluble proteins.

Materials and Reagents

1. Disposable semi-micro cuvettes (BRAND, catalog number: 759015)
2. 50 ml conical tubes (JET biofil, catalog number: CFT011500)
3. Round paint brush, ø 6 mm
4. Pipette tips:
 - 10 µl (Neptune, catalog number: 2340)
 - 200 µl (Eppendorf, catalog number: 0030000870)
 - 1,000 µl (Eppendorf, catalog number: 0030000927)
5. 1.5 ml microtubes (Deltalabs, catalog number: 4092.3N)
6. 7 ml screw cap vials for cell lysis (BioSpec Products, catalog number: 3205)
7. Plastic chromatography column (Bio-Rad, catalog number: 7311550)
8. *Synechocystis* cells expressing a 3x FLAG-tagged protein
9. BG-11 liquid medium and agar plates (Rippka *et al.*, 1979)
10. 100-200 µm glass microbeads (PRECIOSA ORNELA, crystal microbeads B 134)
11. cOmplete protease inhibitor cocktail tablets (Sigma-Aldrich, catalog number: 11836145001)
12. (Optional) Microconcentrators with suitable cut-off filter (e.g., Amicon Ultra-0.5, Sigma-Aldrich)
13. Liquid nitrogen
14. Crushed ice
15. Styrofoam box or equivalent for keeping ice
16. Reverse osmosis (RO) water
17. Deionized water (Merck, catalog number: 1167545000)
18. Methyl alcohol (PENTA, catalog number: 21240-11000)
19. n-dodecyl-β-D-maltoside (DDM; BioChemica, catalog number: A0819,0005)
20. ANTI-FLAG M2 Affinity Gel (Sigma-Aldrich, catalog number: A2220)
21. Proteus Clarification Mini Spin Column (Generon, catalog number: GEN-MSF500)
22. 3x FLAG peptide, 20 mg, > 85% purity (custom synthesis, Genscript, USA)

Notes:

- a. An alternative is to purchase 3x FLAG peptide (Sigma-Aldrich, catalog number: F4799-4MG), however, the custom synthesis is roughly 6 times cheaper.
 - b. Prepare a 3 mg/ml solution in deionized water and store at -20 °C as 100-200 µl aliquots. Thawed peptide can be stored at 4 °C until use.
23. 2-Morpholinoethanesulfonic acid (MES; AppliChem, catalog number: A0689,0250)
 24. MgCl₂·6H₂O (PENTA, catalog number: 16330-31000)
 25. Glycerol (PENTA, catalog number: 14550-11000)
 26. CaCl₂·2H₂O (PENTA, catalog number: 16790-31000)
 27. α-FLAG antibody (Merck, catalog number: F7425)

28. MES buffer (1 L) (see Recipes)
29. 0.5 M MES-NaOH, pH 6.5 (250 ml) (see Recipes)
30. 1 M MgCl₂ (100 ml) (see Recipes)
31. 1 M CaCl₂ (100 ml) (see Recipes)

Equipment

1. Temperature controlled growth facility equipped with an orbital shaker and a light source
2. Laminar flow hood
3. Metallic inoculation loop
4. Gas burner
5. 500 ml Erlenmeyer flasks
6. Round culture flask (10 L)
7. Cimarec™ i Maxi magnetic stirrer (Thermo Fisher Scientific, catalog number: 50088143) and a magnetic stir bar
8. Spectrophotometer (WPA S1200 Spectrawave)
9. Centrifuges and rotors:
 - Centrifuge (Sigma Laborzentrifugen GmbH, model: Sigma 8KS, rotor: 12505-H)
 - Centrifuge (Sigma Laborzentrifugen GmbH, model: Sigma 3K30, rotors: 12155-H [+15 ml conical tube adapters, 13081] and 12158-H)
 - Centrifuge (Eppendorf, model: 5418)
 - Centrifuge (Eppendorf, model: 5415 R)
10. Centrifuge bottles:
 - 500 ml (Nalgene, catalog number: 3141-0500)
 - 80 ml (Sigma Laborzentrifugen GmbH, catalog number: 15080)
 - 27 ml (Sigma Laborzentrifugen GmbH, catalog number: 15032)
11. Deep freezer
12. Regular freezer
13. Mini-Beadbeater-16 (BioSpec Products, catalog number: 607) equipped with a 7 ml vial adapter (BioSpec Products, catalog number: 607TC8)
14. Vortex V-1 Plus (Biosan, catalog number: BS-010203-AAG)
15. 1 ml Hamilton syringe (Hamilton, catalog number: 81330)
16. Automatic pipettes:
 - 0.5-10 µl (Eppendorf, catalog number: 3120000020)
 - 10-100 µl (Eppendorf, catalog number: 3120000046)
 - 100-1,000 µl (Eppendorf, catalog number: 3120000062)
17. Multi-Bio RS-24 rotator (Biosan, catalog number: BS-010117-AAG)
18. Cold hood or cold room, 10 °C
19. A retort stand and a ring clamp

20. 25 ml glass beakers

Procedure

A. Cell culture and harvesting

1. Working in a laminar flow hood, inoculate *Synechocystis* cells using a metallic inoculation loop into five, 500 ml Erlenmeyer flasks, each containing 200 ml BG-11 medium. Grow the cells at 28 °C and 40 µmol/m²/s photosynthetic photon flux density on a rotary shaker set to 150 rpm until the culture reaches mid/end of logarithmic growth phase.
*Note: When the optical density of the culture at 750 nm reaches 0.6-0.8, which corresponds to 9-12 x 10⁷ *Synechocystis* cells/ml.*
2. Collect your cultures (OD₇₅₀ ~0.8) into a 10 L flask and dilute it to 4 L final volume to gain OD₇₅₀ ~0.2 density. Grow the culture until mid/end of logarithmic growth phase with magnetic stirrer (240 rpm) and air bubbling in the same conditions as above.
3. Divide the cell culture into 500 ml centrifuge bottles and pellet the cells by centrifuging at 10,000 x g in a cooling centrifuge (Sigma 8KS, rotor 12505-H) at 4 °C for 20 min.
4. Suspend the cell pellets into a total of 30 ml of MES buffer.

Notes:

- a. All media and equipment used for the preparation of the cell culture should be sterilized. Use the gas burner to sterilize the metal inoculation loop, and autoclave all glassware and growth media prior to use.
- b. Cyanobacterial cultures have negligible absorbance at 750 nm; therefore, the OD at this wavelength is primarily dependent on light scattering (turbidity). Different spectrophotometers differ greatly in their various optical properties, and hence the light scattering value by OD₇₅₀ may vary. Therefore, it is important to establish the OD at which your culture is at the mid/end of logarithmic growth phase by measuring a growth curve *a priori*. Under our standard conditions, the doubling time of *Synechocystis* is approximately 12 h; hence, a culture with OD₇₅₀ = 0.2 will reach desirable OD₇₅₀ in about a day.
- c. In our experience, larger volumes of cyanobacterial cultures (i.e., 4 L) tend to grow slower, probably due to limitations in gas exchange. Therefore, an intensive bubbling of the culture by air is desirable.
- d. At Step A4 you may freeze the cells in liquid nitrogen and store them at -80 °C until use.

B. Cell lysis and thylakoid isolation

1. Pellet the cells in 80 ml centrifuge tubes at 10,000 x g (Sigma 3K30, rotor 12155-H) at 4 °C for 10 min.
2. Discard the supernatant and resuspend the cells into 5 ml of MES buffer using a wet paint brush. Add 30 ml MES buffer to the centrifuge tube and pellet the cells as above.

3. Resuspend the cells into 12 ml of MES buffer and add protease inhibitor: dissolve 1 tablet into 1 ml of deionized water to obtain a 50x stock solution and pipet accordingly to obtain 1x working concentration. You may store the rest of the inhibitor at -20 °C for later use.
4. Add 3 ml of glass beads and 3 ml of cell suspension into four 7 ml screw cap vials (Figure 1A), close the lids and seal them with parafilm (Figure 1B). There should be approximately 1 cm of free space in the tube, you may add some buffer to reach the desired volume.
5. Break the cells for 60 s by Mini-Beadbeater-16. Allow the tubes to cool on ice protected from light for 5 min and repeat the breaking cycle 5 times.
6. After lysis, spin down the beads at 500 x g for 10 s at 4 °C, (Sigma 3K30, rotor 12155-H equipped with adapters for 15 ml conical tubes). Collect the supernatants into a 27 ml centrifuge tube. Wash the beads (Figure 1C) with one bed volume of MES buffer three-to-four times, until the supernatant and the beads appear light green (Figures 1D-1E).
7. Pool all supernatants from Step B6 and pellet the thylakoids by centrifuging at 35,000 x g (Sigma 3K30, rotor 12158-H) at 4 °C for 20 min.
8. At this point, if you are only interested in the isolation of membrane proteins, you may discard the supernatant. Otherwise, collect the supernatant into a new centrifuge tube (marked: SP, 'soluble proteins'), and resuspend the pellet into 10 ml of MES buffer using a wet paint brush (mark the tube: TM, 'thylakoid membranes'). Centrifuge the tubes as above.
9. Collect the supernatant from the 'SP'-tube into a 50 ml conical tube and store it on ice. Discard the supernatants from the 'TM'-tube and resuspend the pellets from both tubes ('SP' and 'TM') into 1/3 of soluble fraction volume of MES buffer. If you are continuing only with the membrane fraction use a total of 10 ml MES buffer and combine the pellets into a 50 ml conical tube.
10. Freeze a 300 µl aliquot of soluble proteins and a 100 µl aliquot of thylakoids in liquid nitrogen and store at -80 °C for further analysis by gel electrophoresis.

Notes:

- a. *After pelleting the cells, the supernatant may appear slightly bluish due to partial breaking of cells after freezing and thawing.*
- b. *From Step B5 onwards it is important to keep the sample cooled (4-10 °C) and protect it from light to prevent the excitation of chlorophyll molecules and the consequent formation of reactive oxygen species. We recommend working under dim green light.*
- c. *Thylakoids are easier to resuspend first into few ml volume, before adding the rest of the buffer.*
- d. *At Step B10 you may also freeze the rest of the soluble protein and thylakoid samples in liquid nitrogen and store them at -80 °C until subsequent solubilization and purification.*

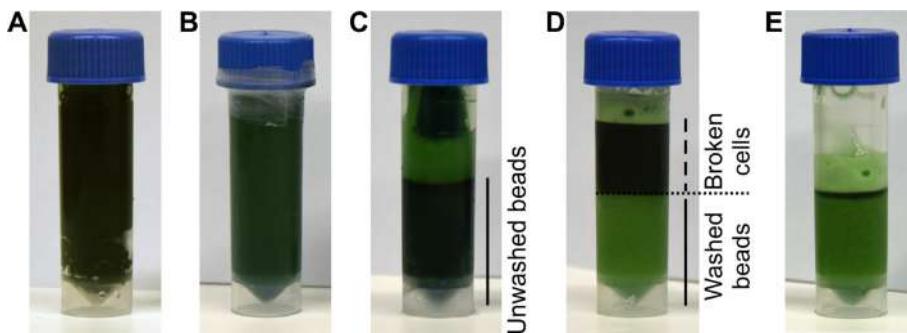


Figure 1. Breaking of *Synechocystis* cells. A. Screwcap tubes are filled with 3 ml of glass beads and 3 ml of cyanobacterial suspension; B. The tubes are sealed with parafilm and cells lysed using a bead beater. C. After breaking cells, the beads are spun to the bottom and the supernatant is collected. D. The beads are washed with MES buffer; E. After each washing step, the supernatant is collected.

C. Thylakoid solubilization

1. Adjust the chlorophyll concentration of the thylakoids to 0.5 µg/µl with MES buffer.
2. Add 1% n-dodecyl β-D-maltoside (DDM) to the thylakoid suspension and incubate the tube for 1 h at 10 °C in the dark (for example, you may cover the tube with aluminum foil) on a rotator mixer set to 10 rpm.
3. Transfer the suspension into a 27 ml centrifuge tube and pellet insoluble material at 46,000 x g (Sigma 3K30, rotor 12158-H) at 4 °C for 30 min.
4. Collect the supernatant into a 50 ml conical tube and discard the pellet.
5. Freeze 100 µl of the solubilized thylakoids in liquid nitrogen and store at -80 °C for further analysis, particularly for the identification of a potential problem with protein solubility.

D. FLAG-affinity purification

1. Wash the purification column with 1 ml of RO-water followed by 1 ml of deionized water.
2. Close the column, then add 1 ml of MES buffer and 600 µl of 1:1 Anti-FLAG-M2 agarose resin using a cut pipette tip (Video 1).



Video 1. Column preparing

3. Open the column and allow the buffer to flow through. Wash the resin with an additional 1 ml of MES buffer (pipet the buffer gently on top of the resin and allow it to flow through; Figure 2A; Video 2).



Video 2. Gentle washing of column

4. Load the protein suspension into the column and collect the flowthrough into a glass beaker (Figure 2B; Video 3).



Video 3. Sample loading

5. Wash the column with 1 ml of MES buffer. Discard the flowthrough.
6. Load the flowthrough from Step D4 to the column for the second time and collect the flowthrough. Repeat the binding once more.
7. Take a 0.5 ml sample from the final flowthrough, freeze it in liquid nitrogen and store at -80 °C for further analysis [Figure 3, flowthrough (FT)].
8. Wash the column with 1 ml of MES buffer [Figure 2C; Figure 3, first wash (W1)].
9. Close the column and add 1 ml of MES buffer: pipet the buffer with high enough speed to mix it thoroughly with the resin (Figure 2D; Video 4) (you may mix the buffer with the resin gently by pipetting up and down with a cut pipette tip, but some resin may stick to the tip and be lost). Open the column and allow the buffer to drain out [Figure 3, second wash (W2)].



Video 4. Vigorous washing of the resin

10. Wash the column with 1 ml of MES buffer without mixing the resin. Repeat this washing step 3 times. In total, the resin is washed by 20 column volumes of MES buffer (Figure 2E).

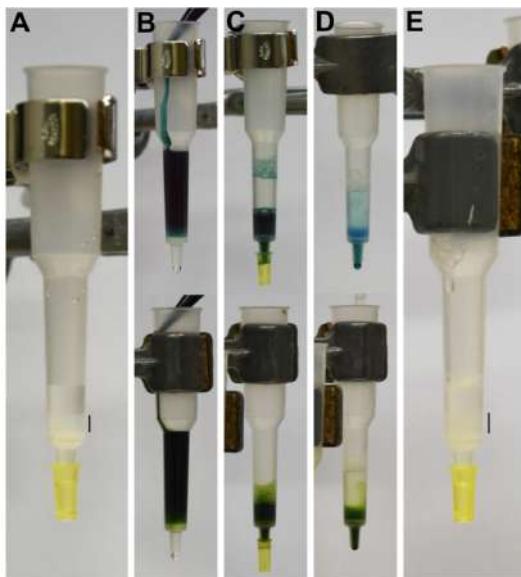


Figure 2. Isolation of 3x FLAG-tagged proteins from *Synechocystis* using affinity chromatography. Individual purification steps for soluble proteins and detergent-solubilized membrane proteins are shown in upper panels and lower panels, respectively. A. Anti-FLAG resin is packed into a plastic chromatography column. B. Soluble protein fraction (upper panel) or detergent solubilized thylakoids (lower panel) are loaded to the column. C. Sample is allowed to flow through the column and the flowthrough is collected. Loading can be repeated several times. D. First wash (W1) is performed by gently pipetting buffer on top of the resin. E. Washing is continued as in panel D until the column is completely washed from excess pigment-proteins. FLAG resin is indicated with a black bar in panels A and E.

11. Resuspend the resin into one bed volume of MES buffer and transfer it into a Proteus Clarification Mini Spin Column.
12. Add 3x FLAG-peptide into a final concentration of 300 µg/ml. Seal the top of the tube with parafilm and cover the whole tube with aluminum foil. Mix in a rotator mixer set to 10 rpm for 30 min at 10 °C.
13. Spin the column at 600 x g (Eppendorf 5415 R) at 4 °C for 3 min. Collect elution 1 into a 1.5 ml microtube and store on ice.
14. Repeat the incubation with FLAG-peptide and centrifuge as above to yield elution 2.
15. The obtained elutions can be pooled and the total elution volume (typically ~800 µl) can be immediately concentrated using microconcentrators (e.g., Amicon Ultra-0.5, Sigma-Aldrich) with a desired cut-off to achieve higher concentration for downstream analysis (protein gel electrophoresis, immunoblotting [see Figure 3, eluate (E)], protein mass spectrometry, single-particle analysis, size-exclusion chromatography, etc.).

Notes:

- a. When working with solubilized thylakoids, use buffers supplemented with 0.04% DDM at all steps. Use buffers without detergent when working with soluble proteins.

- b. FLAG-resin should never be allowed to dry out during the purification.
- c. Thaw the FLAG resin on ice and mix thoroughly before use.
- d. The FLAG resin is stored in buffer as a 50% solution, which means that pipetting 600 μ l of the well mixed solution will result in 300 μ l resin bed volume.
- e. For low abundant proteins, the number of binding steps (Step D6) can be increased.
- f. The stringency of the purification can be altered by increasing the number of washing steps.

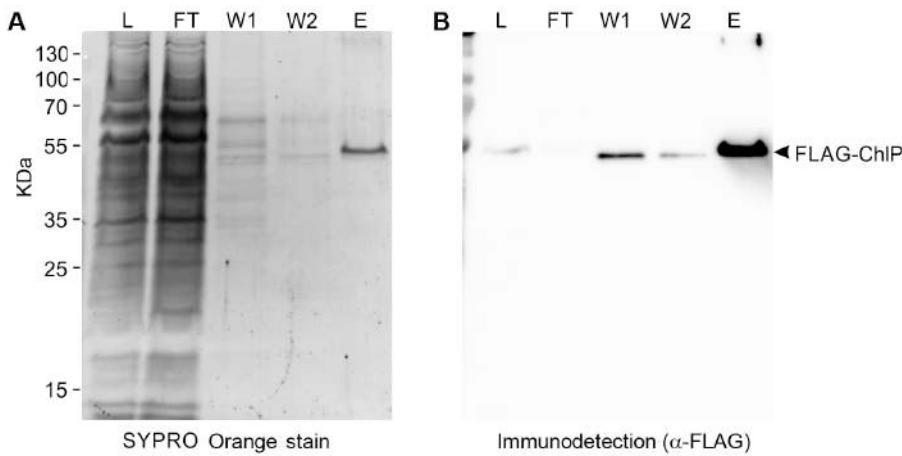


Figure 3. Immunoblot analysis following the purification of geranylgeranyl reductase enzyme tagged with 3x FLAG-tag (FLAG-ChIP). The purification of FLAG-ChIP from *Synechocystis* was performed according to this protocol. Protein suspension that was loaded in Step D4 (L; solubilized thylakoid membranes of 0.5 μ g chlorophyll), the first flowthrough in Step D4 (FT; equal volume to L), the first wash in Step D5 (W1; 1/25 volume of the wash fraction), the second wash in step 6 (W2; 1/25 volume of the wash fraction), and the eluate gained in Step D15 (E; 1/250 volume of total, non-concentrated eluate) were analyzed by SDS gel electrophoresis (A) and by a subsequent immunoblot (B). Prior to Western blotting on a PVDF membrane the gel was stained with SYPRO Orange (Sigma) to detect total proteins (A). Proteins blotted on the membrane were probed with the α -FLAG antibody (Merck) (A); the protein band, representing the isolated FLAG-ChIP, is indicated.

Notes

1. The MES buffer system pH 6.5 containing Mg²⁺ and Ca²⁺ ions used in this procedure has been optimized to maximize the stability of PSII complexes (Dobáková et al., 2009). A different buffer composition may be required for the stability of different protein complexes.
2. The protein concentration in soluble fraction can be assessed e.g., by BCA assay or other standard methods. However, for assessing the concentration of the membrane fraction, it is more convenient to relate to its chlorophyll content [the protein concentration (μ g/ μ l) in *Synechocystis* thylakoids is roughly ten times higher than that of chlorophyll].
3. Measurement of chlorophyll concentration: Pipet 10 μ l of thylakoids into a 1.5 ml microtube and

add 990 µl methanol using a Hamilton syringe. Vortex and incubate the tube in the dark at room temperature for 5 min. Pellet cell debris by centrifuging at 16,800 $\times g$ (Eppendorf 5418) at room temperature for 5 min. Measure the chlorophyll absorbance at 665.2 nm and sample turbidity at 750 nm, and calculate the chlorophyll *a* concentration (µg/µl) of the thylakoids: $((A_{665.2} - A_{750})/\alpha) \times 100 \times 1 \text{ cm}^{-1}$ ($\alpha = 79.95 \text{ L g}^{-1} \text{ cm}^{-1}$; Porra *et al.*, 1989).

4. Always perform a control purification with a wild-type strain to exclude contaminants from your analysis of results. For example, a small amount of *Synechocystis* trimeric photosystem I and tryptophanyl-tRNA synthetase are typical contaminants of our FLAG-tag purifications (Knoppová *et al.*, 2014; Bučinská *et al.*, 2018; Skotnicová *et al.*, 2018).
5. In case the purification fails or the protein yield is very low, make sure that your protein of interest is expressed and not degraded *in vivo* or during the preparation of cellular fractions (perform SDS-PAGE and immunodetection using commercially available anti-FLAG antibody (Merck). A representative SDS gel electrophoresis and subsequent immunoblot analysis of purification (Steps D7-D9) and elution (Step D15) are presented in Figure 3. In the case of membrane proteins, compare the unsolubilized and solubilized fractions to check protein solubility (a detergent with different properties than DDM may be required for the solubility or stability of the desired protein complex). In addition, compare your original SP and/or solubilized TM samples with the obtained flowthrough in Step D7 (Figure 3). The relative amount of your protein in flowthrough should significantly decrease when compared with the loaded material; otherwise it signifies a problem with binding to the resin. For the purification of proteins requiring metal cofactors an EDTA-free protease inhibitor should be used.

Recipes

1. MES buffer (1 L)
25 mM MES-NaOH, pH 6.5 (50 ml of 0.5 M stock)
10 mM MgCl₂ (10 ml of 1 M stock)
10 mM CaCl₂ (10 ml of 1 M stock)
25% glycerol (250 ml)
 - a. Adjust the volume to 1 L with deionized water in a measuring cylinder
 - b. After membrane protein solubilization, use buffer supplemented with 0.04% DDM for all purification steps
2. 0.5 M MES-NaOH, pH 6.5 (250 ml)
24.41 g MES (2-Morpholinoethanesulfonic acid) (AppliChem)
 - a. Dissolve into ~200 ml of deionized water and set pH to 6.5 with 10 M NaOH
 - b. Adjust the volume to 250 ml in a measuring flask
3. 1 M MgCl₂ (100 ml)
20.33 g MgCl₂·6H₂O
Dissolve to deionized water and adjust the volume to 100 ml in a measuring flask

4. 1 M CaCl₂ (100 ml)

14.70 g CaCl₂·2H₂O

Dissolve to deionized water and adjust the volume to 100 ml in a measuring flask

Acknowledgments

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Competing interests

The authors declare no conflicts of interest.

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Determination of Ureides Content in Plant Tissues

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[Abstract] The ureides allantoin and allantoate are the main organic nitrogen compounds transported in several legumes, predominantly from N₂ fixation. Moreover, recent studies point out a remarkable role for allantoin during several stress responses of plants other than legumes. The goal of this protocol is to determine ureides concentration in different plant tissues. Ureides are extracted from plant material by boiling it in phosphate buffer. The allantoin and allantoate present in the supernatants are subjected to alkaline-acidic hydrolysis to glyoxylate. The glyoxylate is converted into glycoxylic acid phenylhydrazone, that is then oxidized to red-colored 1,5-diphenylformazan. The absorbance of supernatants is measured using a spectrophotometer at 520 nm. Ureides concentration can be inferred by using a glyoxylate calibration curve. Ureide quantification of different tissues of *Arabidopsis thaliana* and soybean plants were carried out following this protocol.

Keywords: Allantoin, Allantoate, Ureides, Purine, Plants

[Background] Determination of ureides is important for characterizing N₂ fixation and assimilation in legume plants as well as stress and nutritional responses of non-ureidic plants like *Arabidopsis* (Brychkova *et al.*, 2008; Watanabe *et al.*, 2014; Irani and Todd, 2016 and 2018). The formation of allantoate is also useful for *in vivo* and *in vitro* determination of allantoinase activity (Duran and Todd, 2012). Techniques requiring expensive equipment, such as high-performance liquid chromatography (HPLC), are commonly used for ureide quantification while ethanol extraction is mostly employed for ureide recovery from plant tissues. The present protocol is based on flexible spectrometry that uses low volume of cheap reagents. The use of phosphate buffer instead of ethanol for ureide extraction and quantification greatly improved the reproducibility of the measurements, possibly by decreasing the interfering compounds that abound with the ethanol extraction.

Allantoin and allantoate present in plant extracts are converted to glyoxylate by alkaline-acidic hydrolysis (Vogels and Van der Drift, 1970). To carry out this protocol, three tubes (A, B and C) for alkaline and/or acid hydrolysis are prepared for each sample (Figure 1). The glyoxylate is converted into glycoxylic acid phenylhydrazone and is then oxidized by ferricyanide to form red-colored 1,5-diphenylformazan. The absorbance of supernatants is measured using a spectrophotometer at 520 nm. Allantoin content is obtained by subtracting the levels of glyoxylate resultant of allantoate degradation (tube B) from glyoxylate derived of allantoin alkaline-acid hydrolysis (tube C). Likewise, allantoate content can be inferred by the subtraction of basal glyoxylate levels (tube A) from glyoxylate converted

from allantoate (tube B).

The described protocol was carried out for determine ureide concentration for different tissues, nutritional and stress conditions in *Arabidopsis thaliana* (Lescano *et al.*, 2016 and 2020); and in roots and shoots of nodulating and non-nodulating soybean plants (Muñoz *et al.*, 2016).

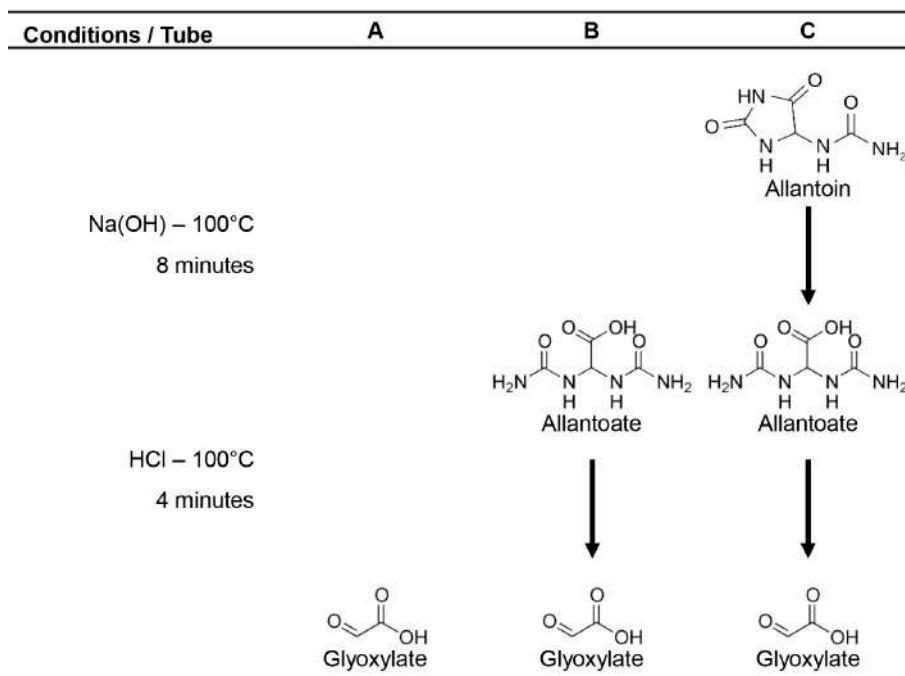


Figure 1. Alkaline-acidic hydrolysis of ureides

Materials and Reagents

21. 1.5 ml microcentrifugue tubes (Eppendorf)
22. 1.5 ml safe-lock microcentrifugue tubes (Eppendorf)
23. 50 ml Falcon tubes
24. Chemical resistant gloves
25. Seeds (e.g., *Arabidopsis*)
26. Growing medium [e.g., for *Arabidopsis* 0.5x MS (1% w/v agar) plates or soil: vermiculite (1:1 mix pots)]
27. Distillated water (dH₂O)
28. (Optional) Liquid air/N₂
29. Monopotassium phosphate (Cicarelli® Laboratories, catalog number: 1057211)
30. Dipotassium phosphate (Cicarelli® Laboratories, catalog number: 1015211)
31. Sodium hydroxide (NaOH) (Cicarelli® Laboratories, catalog number: 843211)
32. Hydrochloric acid (HCl) (Biopack, catalog number: 2000963208)
33. Phenylhydrazine (Merck, catalog number: 107251). Store at room temperature (RT) in the dark
34. Potassium ferricyanide (Anedra, Research AG, catalog number: 7039)

35. Sodium glyoxylate monohydrate (Sigma-Aldrich, Merck, catalog number: G4502)
36. Extraction Buffer (see Recipes)
37. Reaction Buffer (see Recipes)

Equipment

1. Beaker
2. (Optional) Mortar and pestle
3. Plant growth chamber/greenhouse
4. 60-65 °C incubator
5. Analytical balance 0.5/0.0001 g (e.g., Mettler Toledo, model: AL204)
6. Centrifuge at 4°C with microliter rotor (e.g., Thermo Scientific, Sorvall Biofuge Primo R)
7. Ice bath
8. Hot air bath at 100 °C
9. Vortex mixer (e.g., Decalab)
10. Fume hood
11. VIS Spectrophotometer (e.g., Bioamerican Science, model: SP 2000 UV)
12. Spectrophotometric plastic or glass VIS/UV-VIS semi-micro 0.75-1.5 ml cuvettes

Procedure

A. Preparation of plant materials

1. Grow plants in a chamber/greenhouse with the corresponding conditions.

*Note: For grow *Arabidopsis thaliana*, stratify seeds at 4 °C for 2-3 days. Sow seeds on 0.5x MS agar plates or on soil:vermiculite (1:1) mix, and transfer to a growth chamber under a 16 h light/8 h dark photoperiod at 22 °C and a light intensity of 100-150 µmol photons m⁻²s⁻¹.*

2. Collect about 20 mg-0.5 g of plant samples in 1.5 ml safe-lock microcentrifuge tubes.

Notes:

- a. *For Arabidopsis harvest young, senescent and caulinar leaves, buds, flowers, siliques and dry seeds of adult plants (Figures 2A-2F) or collect young seedlings grown in 0.5x MS agar plates (Figure 2G).*
- b. *It is recommended to collect plant samples of similar weights between biological replicates.*

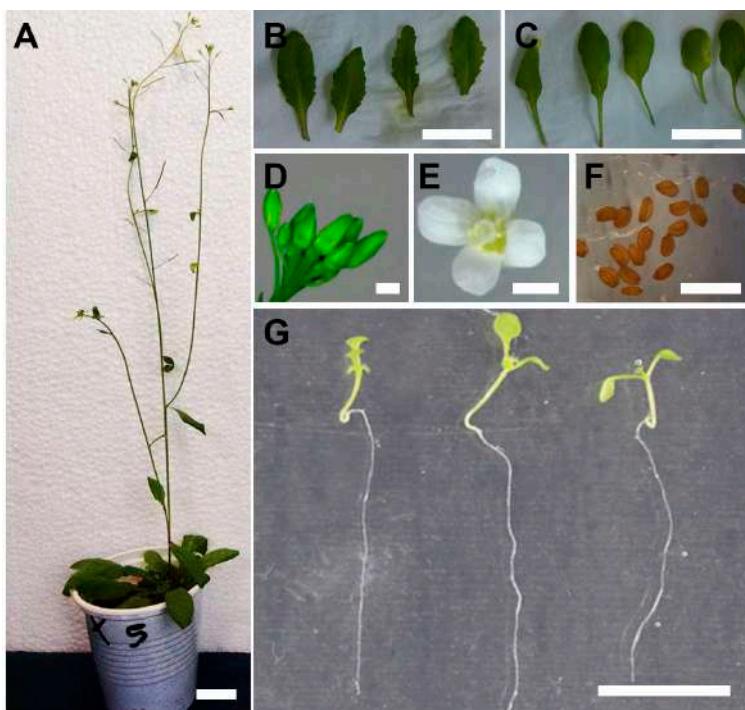


Figure 2. Examples of *Arabidopsis thaliana* tissues used for ureide quantification. (A) 8-week old plant. Details of (B) young leaves, (C) senescent leaves, (D) buds, (E) flowers and (F) seeds are shown. (G) 7 day-old seedlings grown on 0.5x agar plates. Scale bars = 2 cm (A-C, G), = 1 mm (D-F).

3. Put microcentrifuge tubes with open lids in an oven at 60–65 °C overnight.
 4. Weight dry plant samples and put them back in the tubes. Samples can be stored at -20 °C.
- B. Ureide extraction from plant tissues
7. Optional step: ground plant tissues to fine powder with a mortar and a pestle. Transfer the powder to microcentrifuge tubes.
*Note: Grinding plant samples to fine powder depends on the type of tissue and plant species. For example, it is not necessary for ureide extraction from *Arabidopsis*, but could be required for ureide extraction from hard tissues of other plant species, i.e., soybean (Muñoz et al., 2016).*
 8. Add 0.5 ml of Extraction Buffer.
 9. Centrifuge at 17,758 x g for 5 min at 4 °C.
 10. Incubate in a hot air bath at 100 °C for 20 min. Ensure that all the tubes keep well closed.
 11. Transfer to ice bath for 1 min. Ensure that all the tubes keep well closed.
 12. Centrifuge at 17,758 x g for 5 min at 4 °C.
 13. Transfer the supernatant to a new microcentrifuge tube (not necessary safe-lock tube). Samples can be stored at -20 °C for several months, or at 4–8 °C prior to the next step.

C. Alkaline/Acid Hydrolysis

1. Prepare microcentrifuge tubes in which reactions A-C for each sample will be carried out, according the compound/s to be measured (Figure 1). Designate the tubes according which reaction take place in it: A, B and C.

Notes:

- a. *Prepare tubes according to the compound/s to be measured as indicated in Table 1. For example, to measure allantoin and allantoate concentrations in sample #1 tubes can be designated 1A, 1B and 1C.*
- b. *The use of duplicates for each sample is highly recommended.*

Table 1. Tubes preparation

Compound to be measured	A	B	C
Allantoin	-	X	X
Allantoate	X	X	-
Allantoin and allantoate	X	X	X
Total Ureides	X	-	X

2. For the glyoxylate standard curve prepare a fresh serial dilution of 0, 2, 4, 6, 8 and 10 nmols sodium glyoxylate monohydrate in 100 µl Extraction Buffer and follow the steps corresponding to glyoxylate quantification (reaction A). It is not necessary to repeat this step once a proper glyoxylate standard curve is obtained.

Note: It is recommended to use triplicates for each concentration of the glyoxylate standard curve.

Follow the corresponding steps for each reaction as indicated in Table 2. Perform the same steps for the corresponding blank tubes, but adding 100 µl Extraction Buffer instead of the sample (Step C3a). In Steps C3d, C3e, C3h and C3i. ensure that all the tubes keep well closed. Samples can be stored at -20 °C for several months after Step C3j, or maintained at 4-8 °C to continue the protocol immediately.

Note: It is recommended to perform the same reactions simultaneously for the analyzed samples. In the meantime, tubes of other reactions can be stored at 4-8 °C.

Table 2. Ureide hydrolysis

Reagent/Tube	A	B	C
a. Sample	100 µl	100 µl	100 µl
b. dH ₂ O	400 µl	300 µl	200 µl
c. 0.5 N Na(OH)	-	-	100 µl
d. Incubate at 100 °C	-	-	8 min
e. Keep on ice bath	1 min	1 min	1 min
f. 0.65 N HCl	-	-	100 µl
g. 0.15 N HCl	-	100 µl	-
h. Incubate at 100 °C	-	4 min	4 min
i. Keep on ice bath	1 min	1 min	1 min
j. Reaction Buffer	100 µl	100 µl	100 µl

D. Spectrophotometric measurements

Note: Perform HCl work in a fume hood, with the appropriate personal protective equipment.

1. Prepare fresh 3mg/ml phenylhydrazine and 16 mg/ml potassium ferricyanide with dH₂O in Falcon tubes. Mix the tubes content by inverting and shaking vigorously on a vortex. Store these solutions in the dark at RT.
2. Add 100 µl phenylhydrazine 3 mg/ml. Mix on a vortex and incubate for 5 min at RT.
3. Add 500 µl 37% HCl and mix by inversion.
4. Add 100 µl 16 mg/ml potassium ferricyanide and mix by inversion. A pink to red color should be observed.
5. Incubate for 10 min at RT.
6. Measure the absorbance of 1,5-diphenylformazan at 520 nm (Abs₅₂₀) at RT. One value of Abs₅₂₀ is obtained for each reaction tube, namely Abs_{520A}, Abs_{520B}, Abs_{520C}.

Notes:

- a. If the Abs₅₂₀ is above the linear range determined with the standard curve, make a dilution of the sample in 18.5% HCl and measure again immediately. 1/5 and 1/10 dilution are recommended (Dilution factors 5 and 10, respectively).
- b. Make a table to write the obtained data for each sample: Fresh weight, Dry weight, Dilution factor, Abs_{520A}, Abs_{520B}, Abs_{520C}.

Data analysis

1. Determine values of the intercept and slope of the standard curve. A typical glyoxylate standard curve is shown in Figure 3.

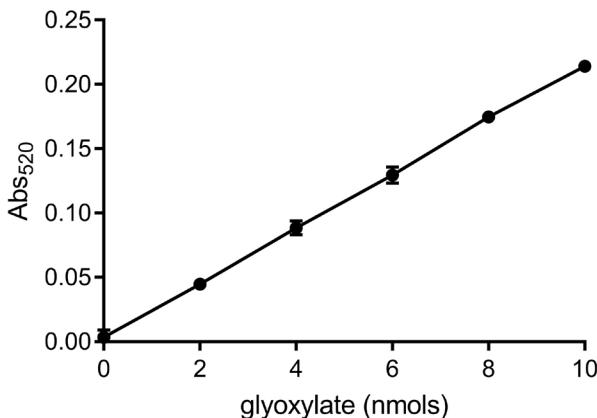


Figure 3. A representative glyoxylate standard curve. The standard curve is generated by performing a serial dilution of sodium glyoxylate monohydrate. Intercept = 0.0006; Slope = 0.0214; R² = 0.9998 (10 nmol point was not taken in account for calculation).

2. Calculate glyoxylate content (G) in tubes A, B and C using the formula [Abs₅₂₀ – intercept]/slope to obtain values G_A, G_B and G_C.
3. Ureide content in the sample can be calculated using the following equation:
total ureide content (nmol) = [G_C-G_A] = [Allantoin content + Allantoate content]
allantoin content (nmol) = [G_C-G_A]
allantoate content (nmol) = [G_B-G_A]
4. Determine ureide concentration of the sample (nmol/mg dry weight) using the formula:
total ureide concentration = [total ureide content (nmol)] × [dilution factor]/[dry weight (mg)]
allantoin concentration = [allantoin content (nmol)] × [dilution factor]/[dry weight (mg)]
allantoate concentration = [total allantoate content (nmol)] × [dilution factor]/[dry weight (mg)]
5. Report ureide concentrations of the analyzed material/s as nmol/mg dry weight (DW), e.g., allantoin and allantoate concentrations in different *Arabidopsis thaliana* tissues are shown in Figure 4. The described protocol was also used for determine changes in ureide concentration in shoots of *Arabidopsis* plants grown under different nutritional and stress conditions (Lescano *et al.*, 2016 and 2020). Allantoin concentration was 18.2 nmol/mg in the roots and 6.3 nmol/mg in the shoots of 2 week-old *Arabidopsis* roots (Lescano *et al.*, 2016, Figure 8). Allantoin and allantoate concentration from xylem sap of 6 week-old *Arabidopsis* were 0.27 ± 0.07 nmol/μl and 0.09 ± 0.08 nmol/μl, respectively. This protocol was also carried out for determine ureide concentration in roots and shoots of nodulating and non-nodulating soybean plants (Muñoz *et al.*, 2016).

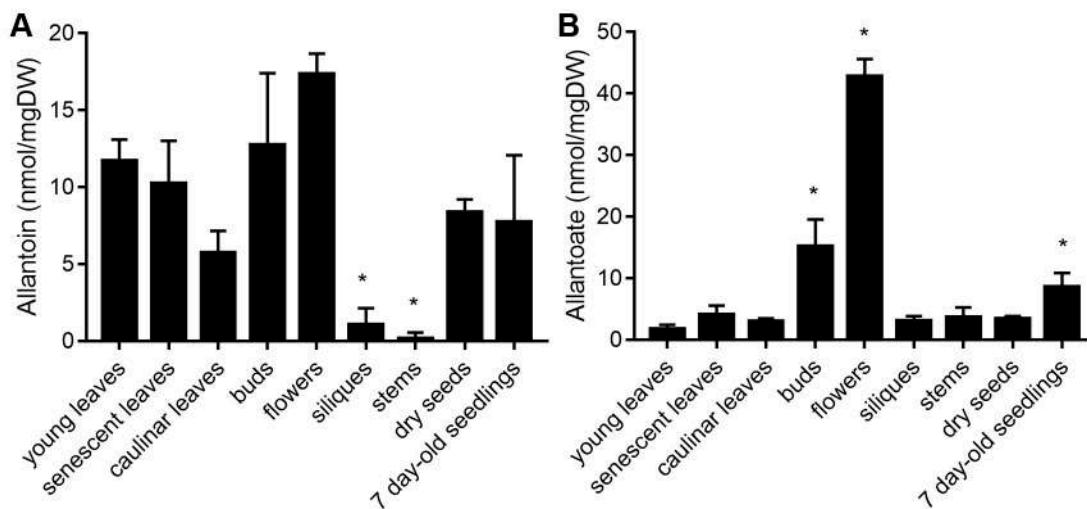


Figure 4. Ureide concentration in different *Arabidopsis* tissues. Allantoin and allantoate content of different organs and tissues of adult plants and 7 day-old whole seedlings. DW = Dry weight. Asterisks indicates significant differences in comparison to young leaves samples ($P < 0.05$, Dunn's test)

Recipes

1. Reaction Buffer (0.4 M potassium phosphate buffer)
 - a. Prepare 500 ml 0.4 M monopotassium phosphate and 500 ml 0.4 M dipotassium phosphate by dissolving the corresponding amount of salts in dH₂O
 - b. Put 200 ml of 0.4 M dipotassium phosphate in a beaker
 - c. Bring the solution to pH 7 by adding as much as needed of 0.4 M monopotassium phosphate. The resulting solution is 0.4 M potassium phosphate buffer. Store Reaction Buffer at 4-8 °C and stock solutions at -20 °C
2. Extraction Buffer (25 mM potassium phosphate buffer)
 - a. Put 31.25 ml Reaction Buffer in a beaker
 - b. Bring the solution to pH 7 with 0.5 N HCl
 - c. And add dH₂O to give a final volume of 500 ml

Acknowledgments

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Competing interests

There are no conflict of interests.

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Gene Expression and Genome Editing Systems by Direct Delivery of Macromolecules into Rice Egg Cells and Zygotes

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[Abstract] Polyethylene glycol calcium (PEG-Ca²⁺)-mediated transfection allows rapid and efficient examination to analyze diverse cellular functions of genes of interest. In plant cells, macromolecules, such as DNA, RNA and protein, are delivered into protoplasts derived from somatic tissues or calli via PEG-Ca²⁺ transfection. To broaden and develop the scope of investigations using plant gametes and zygotes, a procedure for direct delivery of macromolecules into these cells has recently been established using PEG-Ca²⁺ transfection. This PEG-Ca²⁺-mediated delivery into rice egg cells/zygotes consists of four microtechniques, (i) isolation of gametes, (ii) production of zygotes by electrofusion of gametes, (iii) PEG-Ca²⁺-mediated delivery of macromolecules into isolated egg cells or produced zygotes, and (iv) culture and subsequent analyses of the transfected egg cells/zygotes. Because the full protocol for microtechniques (i) and (ii) have already been reported in Toda *et al.*, 2016, microtechniques (iii) and (iv) are mainly described in this protocol.

Keywords: CRISPR/Cas9, Electro-fusion, Gamete, Genome editing, *In vitro* fertilization, PEG-Ca²⁺ transfection, Rice, Zygote

[Background] In angiosperms, fertilization and subsequent developmental events, such as embryogenesis and endosperm development, occur in the embryo sac deeply embedded in ovular tissue (Nawaschin, 1898; Guignard, 1899; Russell, 1992; Raghavan, 2003). Therefore, studies on post-fertilization events in the embryo sac have been conducted predominantly through analyses of *Arabidopsis* mutants or transformants coupled with live-imaging. Alternatively, direct analyses using isolated gametes or zygotes are possible, and an *in vitro* fertilization (IVF) system using the isolated gametes can be used to observe and analyze post-fertilization processes directly (reviewed in Wang *et al.*, 2006). Isolated gametes can be fused electrically, following which the produced zygotes then divide and develop into embryo-like structures and regenerate into plantlets in maize, rice and wheat (Kranz and Lörz, 1993; Uchiumi *et al.*, 2007; Maryenti *et al.*, 2019). These cell manipulation techniques, such as isolation of gametes or zygotes and production of *in vitro* zygotes, make it possible to identify genes expressing in gametes, zygotes and embryos, and fertilization-induced/suppressed genes in zygotes and early embryos have been investigated (Sprunck *et al.*, 2005; Ning *et al.*, 2006; Yang *et al.*, 2006; Steffen *et al.*, 2007; Wang *et al.*, 2010; Wuest *et al.*, 2010; Ohnishi *et al.*, 2011; Abiko *et al.*, 2013; Anderson *et al.*, 2013; Khanday *et al.*, 2019; Rahman *et al.*, 2019; Zhao *et al.*, 2019). However, the functions of most of the genes expressing in a gamete-specific or fertilization-induced/suppressed manner have not been analyzed. Therefore, to broaden and develop the scope of investigations using

gametes and zygotes, a procedure for direct delivery of plasmid DNAs into these cells has been established by PEG-Ca²⁺-mediated transfection. By using this procedure, approximately 30% and 70% respectively of PEG-Ca²⁺-transfected rice egg cells and zygotes showed exogenous and transient expressions of fluorescent proteins from plasmid DNA delivered into the cells (Koiso *et al.*, 2017; Toda *et al.*, 2019). Recently, it was shown that PEG-Ca²⁺-mediated ectopic expression of *OsASGR-BBML1*, an AP-type transcription factor, in rice egg cells resulted in induction of nuclear/cell division of the cells (Rahman *et al.*, 2019). In addition, a genome editing system by direct delivery of CRISPR-Cas9 components into rice zygotes has been developed through PEG-Ca²⁺ transfection. Plasmid DNA harboring a Cas9-gRNA expression cassette or Cas9-gRNA ribonucleoproteins (RNPs) was transfected into zygotes, resulting in the regeneration of plants with targeted mutations in the range of 4%-64% (Toda *et al.*, 2019). In addition to rice, complete IVF systems have also been established in maize and wheat (Kranz and Lörz, 1993; Uchiumi *et al.*, 2007; Maryanti *et al.*, 2019), with all three being the major crop species that provide the majority of human energy intake globally. Taken together, a combination of an IVF system and the present gene expression system described here has enormous potential for efficiently investigating post-fertilization events as well as helping to advance molecular breeding to improve these important crop species.

Materials and Reagents

1. Coverslips (24 x 40 mm) (Thermo Fisher Scientific, catalog number: 125485J) (siliconized at the edges with 3% dichlorodimethylsilane in diethyl ether, Figure 1C)

Note: Non-coated coverslips should be used, as using coated coverslips will lead to the cells becoming attached to the surface of the coverslip.

2. Non-treated plastic dishes with a diameter of 3.5 cm (Iwaki, catalog number: 1000-035)
3. Glass capillary
4. Rice egg cells, sperm cells and zygotes (Figures 1D, 1E and 1G)

*Note: Rice plants (*Oryza sativa L.* cv. *Nipponbare* or cv. *Yukihikari*) are grown in an environmental chamber at 26 °C in a 13/11 h light/dark cycle with a photosynthetic photon flux density of 150-300 µmol photons/m²/s. Under these growth conditions, panicles can be obtained throughout all seasons. Rice egg cells and sperm cells were isolated according to the full protocol described by Toda *et al.* (2016). Rice zygotes are also prepared using electrofusion as described in the same protocol.*

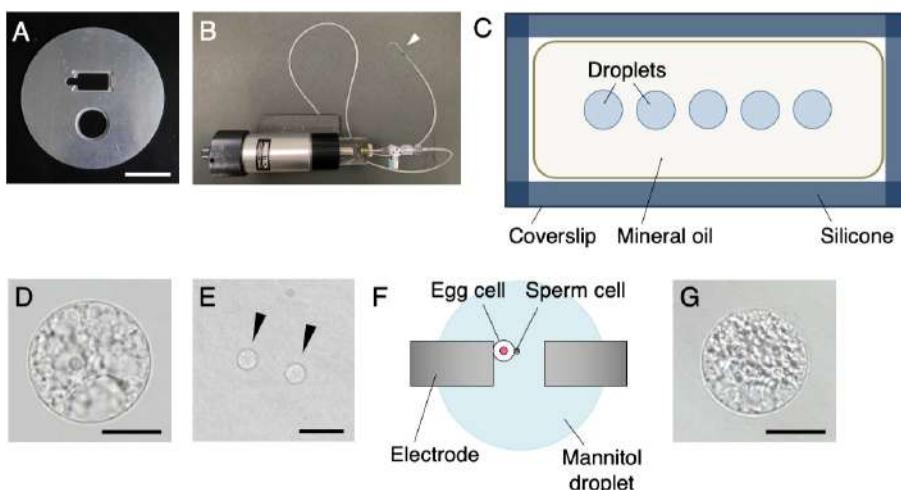


Figure 1. Equipment and materials for cell manipulation (A-C) and preparation of isolated gametes and an *in vitro* zygote (D-G). A. Sliding stage for insertion of a coverslip and a plastic dish. B. Glass capillary-connected handling injector. White arrowhead indicates a glass capillary. C. Image of droplets inside the mineral oil on the siliconized coverslip. D. Isolated rice egg cell. E. Isolated rice sperm cells (black arrowheads). F. Image of electrofusion of an egg cell and a sperm cell using electrodes connected to an electrofusion apparatus. G. Rice zygote produced by electrofusion. Scale bars = 5 cm in A, 20 μ m in D and G, and 15 μ m in E.

5. Feeder cells: rice suspension cell culture (Line Oc, provided by RIKEN Bio-Resource Center, Tsukuba, Japan)

Note: Rice suspension cells, Line Oc, were subcultured once a week according to instructions from RIKEN Bio-Resource Center. No difference in feeder effects between freshly subcultured cells and 1-week-cultured cells has been observed.

6. Mannitol (FUJIFILM Wako Pure Chemical, catalog number: 133-00845)
7. 370 mosmol/kg H₂O (330 mM) mannitol solution (autoclaved)
8. 450 mosmol/kg H₂O (385 mM) mannitol solution (autoclaved)
9. Mineral oil (Sigma-Aldrich, catalog number: M5310-100ML)
10. Absolute ethanol (FUJIFILM Wako Pure Chemical, catalog number: 057-00451)
11. Dichlorodimethylsilane (Tokyo Chemical Industry, catalog number: D0358)
12. Diethyl ether (FUJIFILM Wako Pure Chemical, catalog number: 055-01155)
13. MgCl₂·6H₂O (FUJIFILM Wako Pure Chemical, catalog number: 135-00165)
14. CaCl₂·2H₂O (FUJIFILM Wako Pure Chemical, catalog number: 038-19735)
15. 2-Morpholinoethanesulfonic acid, monohydrate (MES, C₆H₁₃NO₄S·H₂O, Dojindo, catalog number: 349-01623)
16. Polyethylene glycol 4000 (Sigma-Aldrich, catalog number: 81240-1KG)
17. Single guide RNA (synthesized using the GeneArt Precision gRNA Synthesis Kit) (Thermo Fisher Scientific, catalog number: A29377), storage -80 °C

18. Cas9 protein (Thermo Fisher Scientific, GeneArt Platinum Cas9 Nuclease; Thermo Fisher Scientific, TrueCut Cas9 Protein v2, catalog number: A36496), storage -20 °C
 19. The tRNA-based multiplex CRISPR-Cas9 vector (pMgPoef4_129-2A-GFP; accession number LC460477)
 20. Zygote culture medium, storage 4 °C
 21. Regeneration media, storage 4 °C
 22. Rooting media, storage 4 °C
- Note: Recipes for zygote culture medium, regeneration media and rooting media are presented in Toda et al. (2016).*
23. PrepMan Ultra (Thermo Fisher Scientific, catalog number: 4318930)
 24. PrimeSTAR GXL DNA Polymerase (Takara, catalog number: R050A)
 25. ExoSAP-IT Express PCR Cleanup Reagents (Thermo Fisher Scientific, catalog number: 75001.200.UL)
 26. Zero Blunt PCR Cloning Kit (Thermo Fisher Scientific, catalog number: K270020)
 27. MMG solution (see Recipes)
 28. PEG-Ca²⁺ solution (see Recipes)

Note: MMG solution and PEG-Ca²⁺ solution should be freshly prepared, as using old solutions results in decrease of PEG-Ca²⁺ transfection efficiency.

Equipment

1. Forceps
2. Inverted microscope (OLYMPUS, model: IX-71 or IX-73)
3. Sliding stage for the insertion of a coverslip and a plastic dish (Figure 1A)
4. Manual handling injector (PrimeTech, HDJ-M3, Figure 1B)
5. Glass capillaries made from 50 µl aspirator tubes (Drummond Scientific, catalog number: 2-000-050) (Figure 1B)

Note: The procedure for making glass capillaries is presented in Toda et al. (2016).
6. Electrofusion apparatus (Nepa Gene, model: ECFG21)
7. Manipulator (NARISHIGE, model: M-152) with a double pipette holder (Narishige, model: HD-21)
8. Electrodes (Nepa Gene, model: CUY5100Ti100) fixed to the pipette holder (Figure 1F)
9. Millicell-CM inserts, diameter 12 mm (EMD Millipore, catalog number: PICM01250)
10. Environmental chambers
11. Shaker for zygote culture (EYELA, model: Multi Shaker MMS-3020)
12. Osmometer (Gonotec, model: Osmomat 3000 basic)

Software

2. Focas webtool (Focas UI, <http://focas.ayanel.co/>)
3. RNA Folding Form software (mfold web server, <http://unafold.rna.albany.edu/?q=mfold/RNA-Folding-Form2.3>)
4. Parallel Editor in GENETYX software (GENETYX)
5. SnapGene Viewer (SnapGene)

Procedure

A. PEG-Ca²⁺ transfection of rice egg cells with plasmid DNA

1. An outline of procedures for PEG-Ca²⁺ transfection of rice egg cells with plasmid DNA is presented in Figure 2A.
2. As shown in Figures 2C and 2D, prepare 0.5-1 µl droplets of mannitol solution, MMG solution, MMG solution containing plasmid DNA (MMG-DNA, 80-320 ng/µl) and PEG-Ca²⁺ solution (Figures 2C and 2D).

Notes:

- a. *Our study suggested that a MMG-DNA solution containing a higher plasmid concentration (ca. 270-320 ng/µl) generally results in efficient transient expression (Koiso et al., 2017).*
- b. *For preparation of the MMG solution and PEG-Ca²⁺ solution, use mannitol solution adjusted to 370 mosmol/kg H₂O (330 mM) and 450 mosmol/kg H₂O (385 mM) by osmometer for rice egg cells and zygotes, respectively.*
- c. *If the concentration of prepared plasmid DNA sample is low, and the fluid volume of plasmid DNA exceeds that of the MMG solution when you prepare the MMG-DNA solution (80-320 ng/µl), using 2x the MMG solution (double concentration) is recommended.*

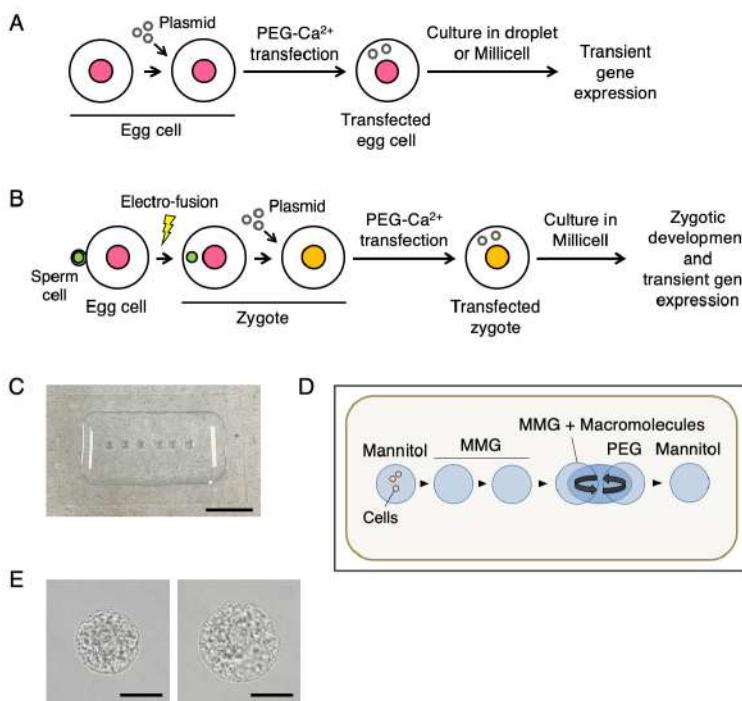


Figure 2. Procedures for PEG-Ca²⁺ transfection of rice egg cells (A) and zygotes (B), preparation of droplets of chemical solutions for PEG-Ca²⁺ transfection (C and D), and rice egg cells upon or after PEG-Ca²⁺ treatment (E). A. Illustration of the procedure for PEG-Ca²⁺ transfection of rice egg cells with plasmid DNA. The transfected egg cells are cultured in a droplet or millicell insert filled with zygote culture medium to observe and analyze the effects of transient gene expression on the cells. B. Illustration of the procedure for PEG-Ca²⁺ transfection of rice zygotes with plasmid DNA. The transfected zygotes are cultured in a millicell insert filled with zygote culture medium to observe and analyze the effects of transient gene expression on zygotic development. C and D. Photo (C) and image (D) of each droplet inside the mineral oil on the siliconized coverslip for PEG-Ca²⁺ transfection. Mannitol, Mannitol solution; MMG, MMG solution; MMG + Macromolecules, MMG solution containing plasmid DNA or ribonucleoprotein; PEG, PEG-Ca²⁺ solution. E. Rice egg cell in the merged droplet of MMG-DNA and PEG-Ca²⁺ solution (left panel) or in mannitol solution (right panel). Egg cell shrank upon PEG-Ca²⁺ treatment, and the size of the egg cell recovered in mannitol solution after the treatment. In A and B, the pink, green and orange circles indicate the egg, sperm and zygotic nuclei, respectively. The yellow lightning bolt and gray rings in A and B indicate the point of electrofusion and plasmid DNAs, respectively. In D, the arrowheads indicate the procedures for transferring cells into each droplet during PEG-Ca²⁺ transfection. The curved arrows above the on MMG + Macromolecules and PEG droplets indicate the process of merging and mixing the two droplets in Step A6. Scale bars = 1 cm in C and 20 μm in E.

3. Transfer approximately three to six egg cells isolated from rice flowers into a 0.5-1 μ l droplet of mannitol solution (370 mosmol/kg H₂O) overlaid with mineral oil on a coverslip using a glass capillary (Figure 2D).

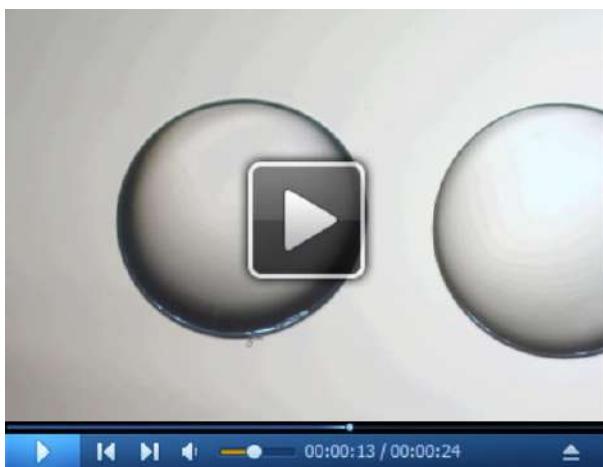
4. Wash the cells twice by transferring them into a MMG solution droplet on a coverslip.
Note: Because the cells tend to attach to the surface of the coverslip in the MMG solution droplet, the manipulation of sucking and releasing cells in the MMG solution droplet should be performed with the tip of the glass capillary positioned slightly above the bottom of the coverslip.
5. Transfer the cells into a droplet of MMG-DNA (Video 1).



Video 1. Rinse rice egg cells with MMG solution by transferring the cells into a MMG droplet, and subsequently transferring the cells into a droplet of MMG-DNA

6. Merge the MMG-DNA droplet, where the cells are transferred, with a droplet of PEG-Ca²⁺ solution using a glass capillary (Figure 2D; Video 2). Then immediately and gently mix the merged droplet using a glass capillary for approximately 1 min.

Note: Although the cells often float in the mixed solution when the MMG-DNA droplet is merged with PEG-Ca²⁺ solution, these cells gradually sink in the solution during incubation.



Video 2. Mixing of the MMG-DNA droplet, where rice egg cells are held, with a droplet of PEG-Ca²⁺ solution using a glass capillary

7. Incubate for 5 min at room temperature. Herein, cell shrinkage can be observed (Figure 2E).

8. After incubation, replace the mixed solution containing the MMG-DNA and PEG-Ca²⁺ solution with the mannitol solution by adding the mannitol solution to the merged droplet and gently mix the droplet using a glass capillary.
9. Suck out approximately half the volume of the mixed solution using a glass capillary (Video 3).



Video 3. Replacement of the mixed MMG-DNA and PEG-Ca²⁺ solution with mannitol solution. Mannitol solution is added to a droplet of the MMG-DNA and PEG-Ca²⁺-mixed solution, and then the droplet is gently mixed using a glass capillary. Thereafter, approximately half of the volume of the solution is removed from the droplet.

10. Repeat Steps A9 and A10 five to six times to reduce the concentration of MMG and PEG-Ca²⁺ in the droplet where PEG-Ca²⁺ treated cells exist. During this process, the shrunken cell recovers its initial round shape (Figure 2E).
11. Transfer the treated cells to fresh mannitol solution (Figure 2D).
12. Wash the cells three to four times by transferring them into droplets of fresh mannitol solution (450 mosmol/kg H₂O) using a glass capillary.
13. Transfer the cells into a droplet of zygote culture medium with several aggregates of the feeder cells (Figure 3A, Nakajima *et al.*, 2010) or into zygote culture medium in a Millicell insert with a drop (approximately 50 µl) of several aggregates of feeder cells (Figure 3B).
Note: Culture of the treated egg cells in the droplet is well suited for short-term observation and analysis (approximately 1-2 days). For long-term culture (more than 2-3 days), a millicell culture is recommended (see Procedure B).
14. Culture the egg cells at 26 °C in the dark without shaking for an appropriate period and conduct analyses of the cells.
*Note: In our study, the transfected egg cells cultured in droplets expressed GFP and DsRed2 fluorescent signals, which are derived from plasmid DNAs transfected into the cells, at approximately 1 day after transfection. Further, when we transfected the rice egg cells with plasmid DNA harboring OsASGR-BBML1, the treated cells cultured in the millicell initiated nuclear/cell division around 2 days after transfection (Koiso *et al.*, 2017, Rahman *et al.*, 2019).*

B. PEG-Ca²⁺ transfection of rice zygotes with plasmid DNA

1. An outline of procedures for PEG-Ca²⁺ transfection of rice zygotes with plasmid DNA is presented in Figure 2B.

2. For PEG-Ca²⁺ transfection, use IVF-produced rice zygotes within 1 h after electrofusion.

Note: As cell wall formation around the plasma membrane of zygotes generally starts after gamete fusion, the early zygotes in which cell walls are thought to be immature should be used for PEG-Ca²⁺ transfection.

3. The PEG-Ca²⁺ transfection of rice zygotes is performed as Steps A2-A11, except that the mannitol solution is adjusted to 450 mosmol/kg H₂O instead of 370 mosmol/kg H₂O.
4. For culture of PEG-Ca²⁺ transfected zygotes in a Millicell insert, place 0.2 ml zygote culture medium in a Millicell-CM insert and put it into a 3.5 cm plastic dish containing 2 ml of the medium. Add 40-60 µl of a rice suspension cell culture into the dish as feeder cells (Figure 3B).
5. Wash the transfected zygotes three to four times by transferring them into droplets of fresh mannitol solution (450 mosmol/kg H₂O) using a glass capillary.
6. Transfer the transfected zygotes onto the membranes of a Millicell insert prepared in Step B4 (Figure 3B).

C. Culture of PEG-Ca²⁺ transfected zygotes

1. After overnight culture of zygotes at 26 °C in the dark without shaking, continue culture with gentle shaking (40 rpm) (Figures 3C-a and 3C-b).
2. At 5 to 7 days after fusion, remove feeder cells by transferring the Millicell dishes containing the embryos into new 35-mm-diameter dishes filled with 2 ml of fresh zygote culture medium and continue culturing as above (Figure 3C-c).
3. After approximately 18-20 days in culture, subculture cell colonies developed from the transfected zygotes in Millicell inserts onto a regeneration medium in plastic dishes by use of a sterilized forcep. Incubate under a 13/11 h light/dark cycle at 28 °C for approximately 15-30 days (Figure 3C-d).
4. Transfer the calli with regenerated shoots into a rooting medium in plastic dishes and culture them under a 13/11 h light/dark cycle at 28 °C for around 15 days (Figure 3C-e).

5. Transfer the resulting plantlets into soil and grow in environmental chambers. If needed, harvest seeds from the regenerated plants (Figure 3C-f).

Note: In our study, approximately 60%-70% of the PEG-Ca²⁺ transfected zygotes grew and regenerated into plantlets.

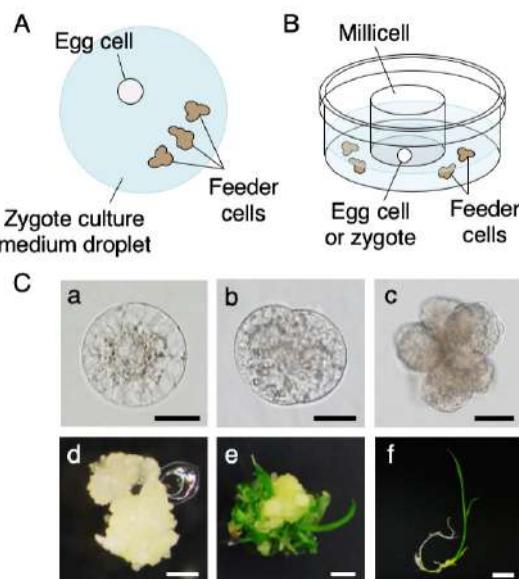


Figure 3. Culture of rice egg cells and zygotes in a droplet (A) and a millicell insert (B), and developmental profiles of rice zygotes (C). A. Illustration of the culture method for rice egg cells in a droplet of zygote culture medium with feeder cells. B. An illustration of the culture method for rice egg cells or zygotes in a millicell insert. C. Developmental profiles of rice zygotes. a. A zygote at 2 h after fusion. b. A globular embryo-like structure at 3 days after fusion. c. A cell mass at 10 days after fusion. d. A white callus at 25 days after fusion. e. A callus with regenerated shoots after 23 days of subculturing in regeneration media (47 days after fusion). f. A plantlet after 17 days of subculturing a callus with regenerated shoots in rooting medium (63 days after fusion). Scale bars = 20 μ m in a and b, 50 μ m in c, 1 mm in d, 2 mm in e and 1 cm in f.

D. Genome editing in rice by direct delivery of CRISPR-Cas9 vector into zygotes

1. Use the Focas webtool for selection of target sequences on genes of interest.
2. In the “on_target_score_calculator” of the Focas webtool, upload the genome file (IRGSP-1.0_genome.fasta) and add target genome sequence information (up to 1 kbp) to obtain candidates of gRNA target sequences (20 mer).
3. Select gRNA target sequences (on_target_score: more than 0.4, without poly T sequence) (Figure 4).

on_target_score							
gene name	DSB position	strand	gRNA target (20 mer)	PAM	On-target score	polyT	Restriction enzyme
no name sequence	53	+	CTCCTCCCTCGCGTCGTTCGT	CGG	0.211539943455		
no name sequence	56	+	CTCCTCCCTCGCGTCGTTCGG	CGG	0.219227243897		
no name sequence	65	+	GCGTTCGTCGGCGCGCATG	CGG	0.18114478677	SphI	
no name sequence	70	+	CGTCGGCGGCATGCGGAA	CGG	0.0627863340548	SphI	
no name sequence	115	+	CGAGAGCTGCTCCCACGACT	CGG	0.370007717216		
no name sequence	122	+	TGCTCCCACGACTCGGATGCG	CGG	0.0451236000767		
no name sequence	125	+	TCCCACGACTCGGATGCCGG	CGG	0.321582190145	Nael	
no name sequence	144	+	GCGGCCGCCCTCAAGTGCAC	CGG	0.348182705884		
no name sequence	163	+	CCGGAAGCTCGTCGTCACC	TGG	0.0385217143706	Sall	

Figure 4. The candidates of gRNA target sequences found by the Focas webtool

4. Put the selected gRNA sequences with a PAM sequence in “CasOT” of the Focas webtool for off-target searches. Select gRNA sequences that show low off-target scores.
5. Predict the secondary structure of single guide RNAs (sgRNAs) containing the selected gRNA sequences using the RNA Folding Form software, available from the mfold web server.

Note: If prediction of the secondary structure of the sgRNAs indicates that a stem-loop structure will be formed through complementary binding in the target-sequence region, avoiding the selected sgRNAs is recommended, as the difference in the targeting efficiency is most likely due to the secondary structure of the guide sequence (Jensen et al., 2017).

6. After selection of the target sequence, prepare a vector harboring a CRISPR-Cas9 expression cassette as below.

*Note: In our study, the tRNA-based multiplex CRISPR-Cas9 vector (pMgPoef4_129-2A-GFP; accession number LC460477) used for both single and multiplex gRNA expression was employed for genome editing. In brief, the vector harbors an *O. sativa* codon-optimized *Streptococcus pyogenes* Cas9 (OsCas9) sequence, fused to GFP via a self-cleaving 2A peptide under the control of the ubiquitin promoter, and the tRNA-gRNA units under a OsU3 gene promoter. The details of the vector construction are described in Toda et al. (2019).*

7. To construct the tRNA-gRNA units for single genome editing, amplify the target sequence by PCR using specific primers to obtain an artificial gene including tRNA and gRNA scaffold sequences.

Note: Information on specific primers is described in Toda et al. (2019).

8. To construct the tRNA-gRNA units for multiplex genome editing, amplify the tRNA-gRNA1-tRNA-gRNA2 sequence by PCR using specific primers to obtain an artificial gene including tRNA and gRNA scaffold sequences.

Note: Information on specific primers is described in Toda et al. (2019).

9. Insert amplified target sequences or tRNA-gRNA fragments into the *Bsa*I site of the multiplex CRISPR-Cas9 vector using Golden Gate Cloning methods (New England Biolabs).

10. Deliver the produced CRISPR-Cas9 vector into rice zygotes by PEG-Ca²⁺ transfection according to Procedure B.

11. Culture the transfected zygotes as described in Procedure C.

- E. Genome editing in rice by direct delivery of Cas9-gRNA ribonucleoproteins into zygotes
1. Select target sequences on each target gene according to Steps D1-D5.
 2. Synthesize and purify sgRNA containing the target sequence using the GeneArt Precision gRNA Synthesis Kit according to the manufacturer's instructions (Thermo Fisher Scientific).
 3. Premix Cas9 protein (1 μ l) and *in vitro* transcribed sgRNA (2-3 μ g) in a total volume of 1.5-2.1 μ l and incubate the premix solution at room temperature for 10 min.
 4. After incubation, gently mix the solution containing the CRISPR RNPs with 10 μ l of MMG solution (MMG-RNP).
 5. Perform PEG-Ca²⁺ transfection of rice zygotes as Procedure B except for the use of MMG-RNP instead of MMG-DNA.
 6. Culture the transfected zygotes as described in Procedure C.
- F. Validation of targeted mutagenesis in the plants regenerated from PEG-Ca²⁺-transfected zygotes by genomic DNA PCR and sequencing of the target sites
1. Cut small pieces of the leaves (2-3 mm²) of rice plants regenerated from zygotes previously PEG-Ca²⁺-transfected with a CRISPR-Cas9 plasmid or RNP.
 2. Prepare genomic DNAs from the cut leaves using PrepMan Ultra according to the manufacturer's instructions.
- Note: In our study, a cut plant leaf is put into a 40 μ l solution of PrepMan Ultra and heated at 95 °C for 20 min. Thereafter, the heated sample is diluted with 100 μ l sterilized water for subsequent genomic DNA PCR.*
3. Amplify PCR fragments containing the target site using PrimeSTAR GXL DNA polymerase with genomic DNAs and a primer set for each target.
 4. After genomic DNA PCR, purify the amplified PCR fragments using ExoSAP-IT Express PCR Cleanup Reagents, and determine the nucleotide sequence of the PCR fragment.
 5. Confirm targeted mutagenesis of the genome-edited plants by Parallel Editor program in GENETYX software. Sanger sequencing chromatograms at target sites of genes on genomic DNA should also be confirmed using SnapGene Viewer.
 6. When the sequencing results show heterologous target mutations, the amplified PCR fragments in Step F3 are subcloned into the pCR-Blunt vector using the Zero Blunt PCR Cloning Kit, and sequence of plasmid inserts from four or more independent clones of transformed *E. coli* are determined.
 7. Confirm targeted mutagenesis of genome-edited plants as described in Step F5.
- Note: A flow chart of the present genome editing procedures are presented in Figure 5.*

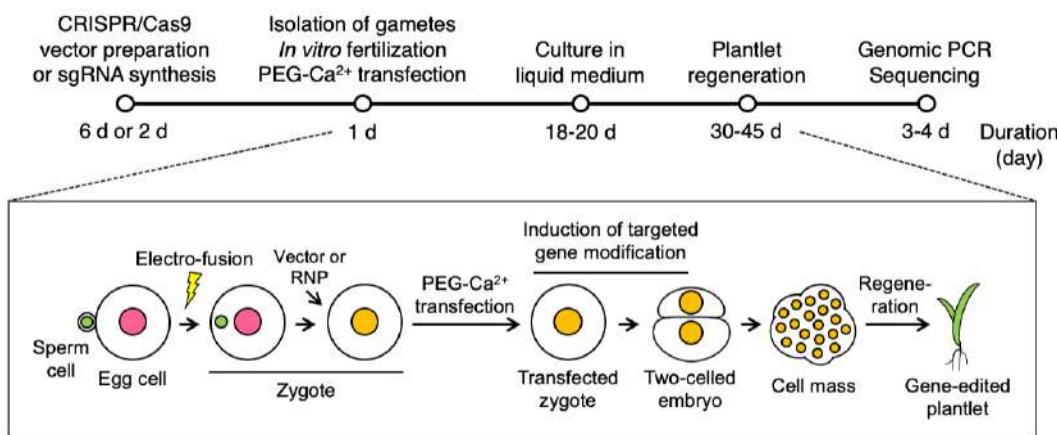


Figure 5. A Flow chart of the procedures for genome editing in rice by direct delivery of the CRISPR-Cas9 vector or RNP in zygotes. The upper section shows the duration in days of each procedure. Pink, green and orange circles indicate the egg, sperm and zygotic nuclei, respectively. The yellow lightning bolt and green leaf shapes indicate the point of electro-fusion and plant leaves, respectively.

Recipes

1. MMG solution
4 mM MES-KOH (pH 5.7)
15 mM MgCl₂
In mannitol solution (370 mosmol/kg H₂O for rice egg cells; 450 mosmol/kg H₂O for rice zygotes)
2. PEG-Ca²⁺ solution
30% PEG4000
100 mM CaCl₂
In mannitol solution (370 mosmol/kg H₂O for rice egg cells; 450 mosmol/kg H₂O for rice zygotes)

Acknowledgments

This protocol was adapted from Uchiumi *et al.* (2007), Okamoto (2011), Toda *et al.* (2016 and 2019), Koiso *et al.* (2017), Toda and Okamoto (2020). This work was supported in part by a research fellowship from the JSPS (Grant-in-Aid for JSPS Research Fellow; grant no. 19J12912 to ET), MEXT KAKENHI (Grant-in-Aid for Scientific Research on Innovation Areas; grant nos. 17H05845 and 19H04868 to TO).

Competing interests

ET and TO are co-inventors on a patent application covering the protocols described in this paper.

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Efficient Agrobacterium-mediated Transformation of the Elite–*Indica* Rice Variety Komboka

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[Abstract] Genetic transformation is crucial for both investigating gene functions and for engineering of crops to introduce new traits. Rice (*Oryza sativa* L.) is an important model in plant research, since it is the staple food for more than half of the world's population. As a result, numerous transformation methods have been developed for both *indica* and *japonica* rice. Since breeders continuously develop new rice varieties, transformation protocols have to be adapted for each new variety. Here we provide an optimized transformation protocol with detailed tips and tricks for a new African variety Komboka using immature embryos. In Komboka, we obtained an apparent transformation rate of up to 48% for GUS/GFP reporter gene constructs using this optimized protocol. This protocol is also applicable for use with other elite *indica* rice varieties.

Keywords: *Agrobacterium*-mediated transformation, *Indica* rice, *Oryza sativa*, Elite variety, Rice transformation, Plant regeneration

[Background] Various methods for genetic transformation of plants have been developed, e.g. PEG-mediated transfection of protoplasts (Shimamoto *et al.*, 1989; Datta *et al.*, 1992), biolistic transformation (Christou *et al.*, 1991) and *Agrobacterium*-mediated transformation (Slamet-Loedin *et al.*, 2014). *Agrobacterium*-mediated transformation is one of the most widely used methods to introduce DNA into plants (van Wordragen and Dons, 1992). This method has been used intensively for research and has become a key prerequisite for biotechnology. It has gained in importance since the development of new breeding technologies such as genome editing (Char *et al.*, 2019). For crops, such as rice, genetic transformation could also be used to develop new genetic variations for plant breeding, for example, creating new disease- or insect-resistant lines (Cheng *et al.*, 1998; Helliwell and Yang, 2013; Oliva *et al.*, 2019). Also for rice, *Agrobacterium*-mediated transformation is the most popular method to transfer T-DNA into plant genomes (Hiei and Komari, 2008). Currently, there are multiple protocols for *japonica* and *indica* rice transformation using calli induced from mature seeds or immature embryos (Hiei and Komari, 2006 and 2008; Toki *et al.*, 2006; Nishimura *et al.*, 2006; Sahoo *et al.*, 2011; Sahoo and Tuteja, 2012; Slamet-Loedin *et al.*, 2014; Sundararajan *et al.*, 2017). It is convenient to use mature

seeds for transformation because they are available throughout the year and can be stored, although this method is predominantly used for *japonica* varieties. Methods that involve transformation of rice immature embryos generally yield higher transformation rates compared to mature seeds (Hiei and Komari, 2008; Slamet-Loedin et al., 2014). Overall, *japonica* varieties such as Kitaake and Nipponbare are apparently easier to transform, compared to *indica* rice such as IR64 or Ciherang-Sub1 (Oliva et al., 2019). For *japonica* varieties, highly efficient transformation can be obtained using calli induced from mature seeds with rates of 50-60% (Li et al., 2015). For *indica* varieties, despite some efforts to increase transformation rate using mature seeds, immature embryos-derived calli are still the tissue of choice for transformation. Notably, transformation efficiency is highly variety-dependent and it is necessary to optimize transformation protocols for each variety.

Komboka (IR 05N 221) is a new elite variety generated by IRRI and released in Tanzania by the National Rice Research Program-KATRIN Research Centre and IRRI-Tanzania ('Komboka' = 'liberated') (Kitilu et al., 2019). Komboka is high yielding (8.6 t ha^{-1}), semi-aromatic with good grain quality, tolerant to blast and well adapted to upland and lowland areas. In the protocol reported here, we describe detailed steps for the stable transformation of Komboka immature embryos which produce transgenic plants within four months and with a high apparent transformation rate of up to 48%. This protocol was developed by combining and modifying published protocols from Slamet-Loedin et al., 2014 and Hiei and Komari, 2008 which were used to transform different *indica* rice varieties such as IR64, Ciherang-sub1 (Oliva et al., 2019), therefore, in principle this protocol can likely be adapted for transformation of other *indica* rice varieties. In this protocol, we highlighted all details, tips and tricks that are essential for setting up transformation protocols for other elite varieties.

Figure 1. Flow chart and timeline for *Agrobacterium*-mediated transformation of immature embryos of rice var. Komboka (*indica*) which takes about four months to generate transgenic lines. Briefly, the process starts with growing rice plants to the flowering stage under controlled greenhouse conditions at $30^{\circ}\text{C} \pm 2^{\circ}\text{C}$ during the day and $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ during the night, 50-70% relative humidity. Light conditions in the glass greenhouse are determined by natural daylight and additional lamplight (8/16 day/night photoperiod). Daylength varies in the course of the seasons due to the location of our greenhouse along latitude (latitudes and longitudes of Düsseldorf, Germany $51^{\circ}11'31.2''\text{N}$ $6^{\circ}47'40.1''\text{E}$). Under these conditions, Komboka required 13-14 weeks till booting stage and additional 2-3 weeks to flower. 8-12 days post-pollination, rice panicles can be harvested for immature embryos isolation. Selecting the proper stage of seeds for immature embryos isolation is crucial. Immature embryos should be in the late milky stage (Video 1), with a size of 1.3-1.8 mm. After dehusking the seeds and isolating immature embryos (Videos 2 and 3; Figure 2), the isolated immature embryos were moved, as a whole, onto co-cultivation medium. Right after the isolation step immature embryos were co-cultivated with *Agrobacteria* harboring constructs-of-interest for seven days. Afterwards, germinated immature embryos were cleaned from *Agrobacteria* with sterile filter papers and shoots were excised. The immature embryos were then transferred onto resting/recovery medium without hygromycin B for five days. After resting, immature embryos were moved to two rounds of selection with 30 mg/L hygromycin B, each for 10 days. After the second selection step, microcalli appeared on brownish maternal immature embryos. Microcalli

were carefully separated from the maternal tissues and transferred onto selection medium (30 mg/L hygromycin B) for the third selection round. In cases that the immature embryos did not produce microcalli after the first two rounds of selection, immature embryos were moved to an additional selection round (Supplementary Selection 2) of 10 days each. Resistant and multiplied microcalli after the third selection were transferred onto pre-regeneration medium (30 mg/L hygromycin B) for 1 or 2 rounds of growth for 10 days. Only greening calli were transferred onto regeneration medium (30 mg/L hygromycin B) for 14 days. When small rice plants (plantlets) were regenerated from the calli and reached about 5 cm in height, they were transferred onto rooting medium without hygromycin B for 14 days. Only well-developed plantlets with strong root systems were planted in pots. Pots were submerged in larger buckets (5 L).

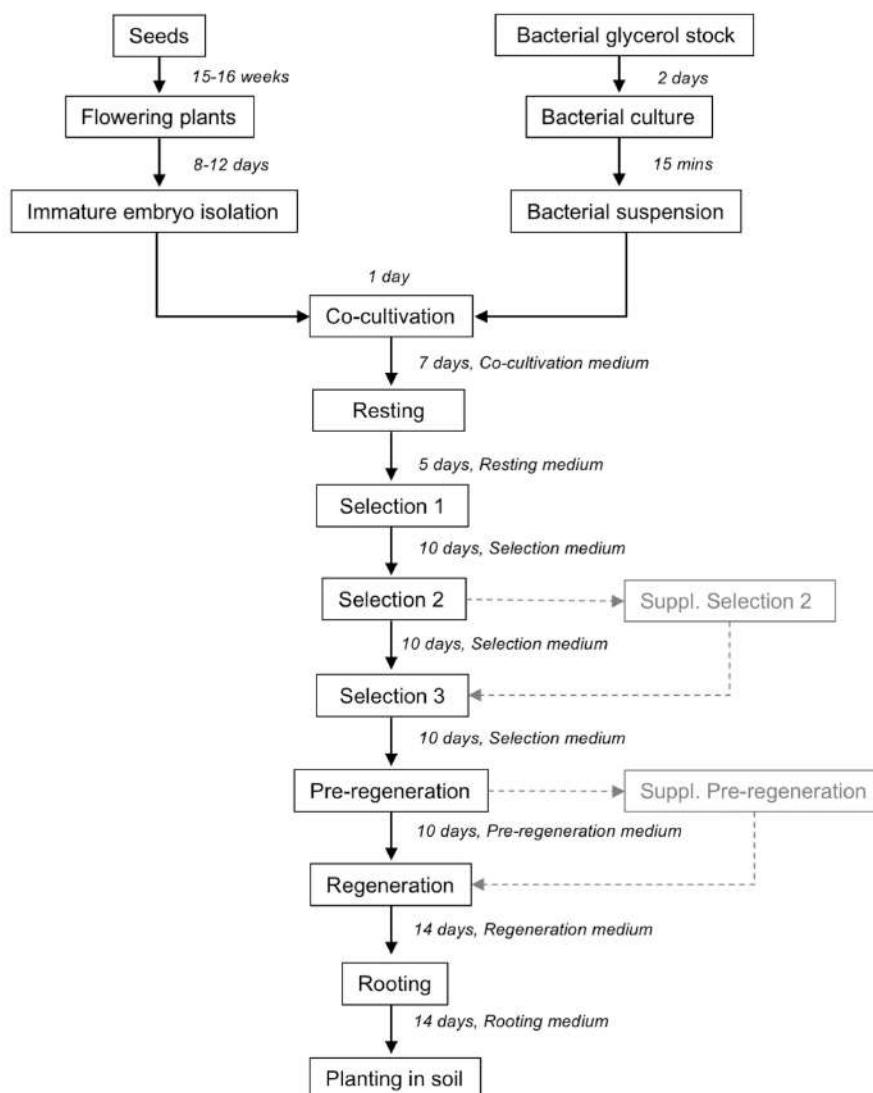


Figure 1. Flow chart describing Agrobacterium-mediated transformation using immature embryos of rice variety Komboka

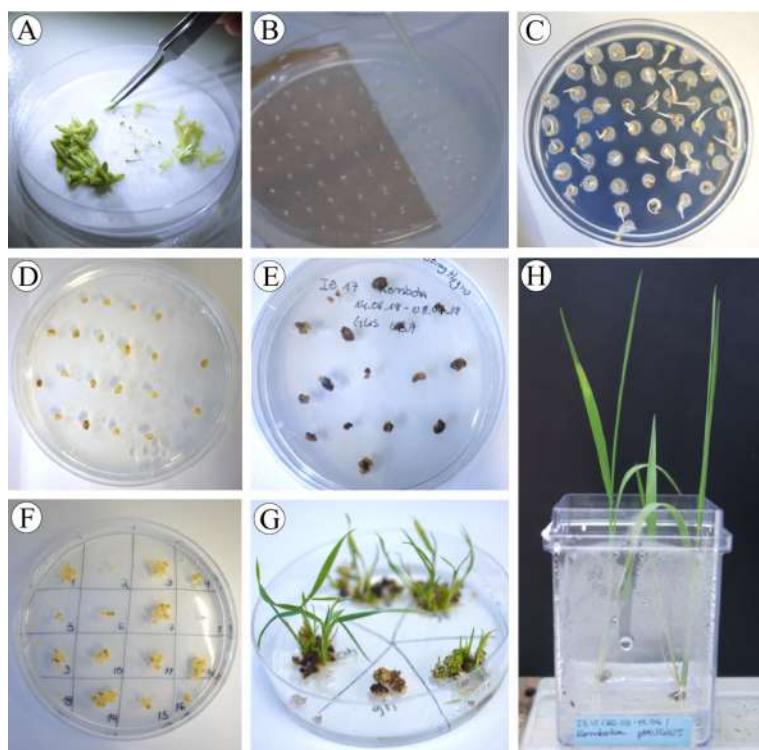


Figure 2. Steps in Agrobacterium-mediated transformation of immature embryos from rice variety Komboka. A. Immature embryo isolation. B. Agrobacterium infection. C. Co-cultivation. D. Resting phase. E. Selection phase. F. Pre-regeneration phase. G. Regeneration phase. H. Rooting phase.

Below, we provide detailed lists of chemicals, equipment, the transformation steps with important notes, as well as all templates for media preparation.

Materials and Reagents

Consumables

1. Filtropur BT 100 bottle top filter (volume: 1000 ml; membrane: PES; diameter: 90 mm; pore size: 0.2 µm for sterile filtration) (Sarstedt, catalog number: 83.3942.101); use to filter stock solutions and for single use only
2. Filtropur BT 25 bottle top filter (volume: 250 ml; membrane: PES; diameter: 90 mm; pore size: 0.2 µm for sterile filtration) (Sarstedt, catalog number: 83.3940.101); use to filter stock solutions and for single use only
3. Filtropur BT 50 bottle top filter (volume: 500 ml; membrane: PES; diameter: 90 mm; pore size: 0.2 µm for sterile filtration) (Sarstedt, catalog number: 83.3941.101); use to filter stock solutions and for single use only
4. Glass test tubes, 14 x 16 mm; 21 ml (DURAN®, catalog number: 26 130 21)
5. Gene-Pulse Cuvettes, 0.2 cm gap (Bio-Rad, catalog number: 1652086), single use only
6. Petri dishes, extra deep, disposable, Lab-tek™ (diameter: 100 mm; height: 26 mm; sterile)

- (Supplier: Thermo Scientific, VWR, catalog number: NUNC4031); use to pour Regeneration medium, single use only
7. Petri dishes, deep, with 3 vents (diameter: 92.3 mm; height: 20 mm; sterile) (Supplier: Greiner Bio One, VWR, catalog number: 391-0493); use to pour Pre-regeneration medium, single use only
 8. Petri dishes, with cams (diameter: 92 mm; height: 16 mm; sterile) (Sarstedt, catalog number: 82.1473); use to pour YEP, Co-cultivation, Resting and Selection media, single use only
 9. Qualitative filter papers, standard grades, grade 1 and 1 V, Whatman® (diameter: 85 mm) (VWR, catalog number: 516-0593)
 10. Reaction tubes, 1.5 ml (Sarstedt, catalog number: 72.690.001)
 11. SafeSeal reaction tube, 2 ml (Sarstedt, catalog number: 72.695.500)
 12. Scalpel blades (type 11 for scalpel handles using the BAYHA interlocking system; non-sterile) (Behr Labor-Technik, catalog number: 9409911)
 13. Screw cap tube, 50 ml (Sarstedt, catalog number: 62.547.254)
 14. Screw cap tube, 15 ml (Sarstedt, catalog number: 62.554.502)
 15. Serological Pipette, 10 ml (Sarstedt, catalog number: 86.1254.001), single use only
 16. Serological Pipette, 25 ml (Sarstedt, catalog number: 86.1685.001), single use only
 17. Serological Pipette, 5 ml (Sarstedt, catalog number: 86.1253.001), single use only
 18. Stretch foil (Stretchplus; 300.0 m x 40.0 cm; foil thickness: 7 µm)
 19. Surgical tape (3M™ Micropore™; Width: 1.25 cm)
 20. Syringe (Injekt®; 20 ml; Luer lock) (Braun, catalog number: 4606205V), single use only
 21. Syringe filtration unit Filtropur S 0.2 (membrane: PES; filtration surface: 5.3 cm²; pore size: 0.2 µm for sterile filtration) (Sarstedt, catalog number: 83.1826.001); use to filter phytohormones and antibiotic solutions, single use only
 22. Tissue culture vessel, Magenta™GA-7 (Supplier: Sigma-Aldrich; 77 x 77 x 97 mm) (VWR, catalog number: SAFSV8505); use to pour 'Roo' medium, reusable
 23. Tooth pick made of wood (6.8 cm) (Pap Star, catalog number: 12736)

Biological material

1. Komboka seeds (IR05N221, L17WS.06#24)

Komboka seeds were kindly provided by the International Rice Research Institute (IRRI, Philippine) under a special material transfer agreement (SMTA-MLS).

Komboka seeds were sown directly into soil in round, 2 L pots (13.2 cm height and 16.7 cm diameter), one plant per pot. A soil mixture of profile porous ceramic (PPC) greens grade and 'Arabidopsis soil' with a ratio of 1:1 is used as a standard soil for rice cultivation. See [Table S1](#) for the details of soil composition. As a slowly released fertilizer, 4 g of Osmocote Exact 3-4 M (16% N, 3.9% P, 10% K, 1.2% Mg, 0.45% Fe, 0.06% Mn, 0.02% B, 0.05% Cu, 0.02% Mo, 0.015% Zn) (ICL/SF UK) was added in 1 L soil. In addition, plants were fertilized weekly from the 2nd week and biweekly from the 6th week after germination using Peters Excel (14% N, 6% P, 14%

K, 6.5% Ca, 2.5% Mg, 0.12% Fe, 0.06% Mn, 0.02% B, 0.015% Cu, 0.01% Mo, 0.015% Zn) (ICL/SF UK). Fertilization was terminated when plants reached the flowering stage. Rice plants in 2 L pots filled with soil were submerged into a 5 L buckets filled with water, so that the inner 2 L pot is under the waterline. Water can remain in the bucket till rice plants get seeds, no exchange needed. Alternatively, 2 L rice pots can also be placed in 60 x 40 x 6 cm trays and filled with water to the upper edge. In this case, water needs to be exchanged biweekly.

Rice plants were grown in the glass house with natural daylight and additional lamplight of 8/16 day/night photoperiod, however not strictly required, with day temperature of 30 °C ± 2 °C and night temperature of 25 °C ± 2 °C. The relative humidity was between 50-70% which was manually controlled by spraying water on the greenhouse floor. Plants were grown under a photosynthetic active radiation (PAR) or photosynthetic photon flux density (PPFD) of 200 µmol m⁻²s⁻¹ with PPFD-blue of 40 µmol m⁻²s⁻¹, PPFD-green of 70 µmol m⁻²s⁻¹ and PPFD-red of 80 µmol m⁻²s⁻¹.

Chemicals

Note: Chemical batches and brands are important factors that may affect transformation success. We found that the indistinctive use of alternative chemical brands often leads to failure when trying to transform Komboka for the first time. Therefore, we highly recommend to use the exact chemicals specified here (brands and catalog numbers), in particular when setting up a new transformation protocol. Make sure to store the chemicals in the required conditions and do not use products after the expiry dates. We declare no competing interest regarding chemicals or instrument choice.

1. 1-Naphthaleneacetic acid, C₁₂H₁₀O₂ (Sigma-Aldrich, catalog number: N0640; CAS number: 86-87-3), store at RT
2. 2,4-Dichlorophenoxyacetic acid, Cl₂C₆H₃OCH₂CO₂H (Sigma-Aldrich, catalog number: D7299; CAS number: 94-75-7), store at RT
3. 3',5'-Dimethoxy-4'-hydroxyacetophenone, acetosyringone, HO-C₆H₂(OCH₃)₂COCH₃ (Sigma-Aldrich, catalog number: D134406; CAS number: 2478-38-8), store at 4 °C
4. 6-Benzylaminopurine, C₁₂H₁₁N₅ (Sigma-Aldrich, catalog number: B3408; CAS number: 1214-39-7), store at RT
5. Agarose type I, low EEO (Sigma-Aldrich, catalog number: A6013; CAS number: 9012-36-6), store at RT
6. Ammonium nitrate, NH₄NO₃ (Sigma-Aldrich, catalog number: A3795; CAS number: 6484-52-2), store at RT
7. Ammonium sulfate, (NH₄)₂SO₄ (Sigma-Aldrich, catalog number: A3920; CAS number: 7783-20-2), store at RT
8. Bacto agar, dehydrated (Fischer Scientific, catalog number: 214050), store at RT
9. Bacto beef extract, desiccated (Fischer Scientific, catalog number: 211520), store at RT
10. Bacto peptone, dehydrated (Fischer Scientific, catalog number: 211677), store at RT

11. Bacto yeast extract (Fischer Scientific, catalog number: 212750), store at RT
12. Boric acid, H₃BO₃ (Sigma-Aldrich, catalog number: B6768; CAS number: 10043-35-3), store at RT
13. Calcium chloride dihydrate, CaCl₂·2H₂O (Sigma-Aldrich, catalog number: C7902; CAS number: 10035-04-8), store at RT
14. Carbenicillin disodium, C₁₇H₁₆N₂Na₂O₆S (Duchefa, catalog number: C0109; CAS number: 4800-94-6), store at 4 °C
15. Cefotaxime sodium, C₁₆H₁₆N₅O₇S₂Na (Duchefa, catalog number: C0111; CAS number: 64485-93-4), store at 4 °C
16. Chloro cleaner, e.g., DanKlorix® original (2.8 g/100 ml sodium hypochlorite), store at RT
17. Cobalt (II) chloride hexahydrate, CoCl₂·6H₂O (Sigma-Aldrich, catalog number: C2911; CAS number: 7791-13-1), store at RT
18. Copper (II) sulfate pentahydrate, CuSO₄·5H₂O (Sigma-Aldrich, catalog number: C3036; CAS number: 7758-99-8), store at RT
19. D-(+)-Glucose, C₆H₁₂O₆ (Sigma-Aldrich, catalog number: G7021; CAS number: 50-99-7), store at RT
20. D-Mannitol, C₆H₁₄O₆ (Sigma-Aldrich, catalog number: M1902; CAS number: 69-65-8), store at RT
21. D-Sorbitol, C₆H₁₄O₆ (Carl Roth, catalog number: 6213.1; CAS number: 50-70-4), store at RT
22. Difco™ casamino acids, vitamin assay (Thermo Fischer, catalog number: 228820), store at RT
23. Dimethyl sulfoxide (DMSO) (Fisher Scientific, catalog number: D/4121/PB15; CAS number: 67-68-5), store at RT
24. Ethylenediaminetetraacetic acid disodium salt dihydrate, C₁₀H₁₄N₂Na₂O₈·2H₂O (Sigma-Aldrich; catalog number: E6635; CAS number: 6381-92-6), store at RT
25. GELRITE™ (Duchefa, catalog number: G1101; CAS number: 71010-52-1), store at RT
26. Glycerol, SOLVAGREEN® ≥ 98 %, anhydrous, Ph.Eur., C₃H₈O₃ (Carl Roth, catalog number: 7530.1; CAS number: 56-81-5), store at RT
27. Glycine, NH₂CH₂COOH (Sigma-Aldrich, catalog number: G8790; CAS number: 56-40-6), store at RT
28. Hygromycin B solution, CELLPURE® 50 mg/ml, sterile, C₂₀H₃₇N₃O₁₃ (Carl Roth, catalog number: CP12.2; CAS number: 31282-04-9), store at 4 °C
29. Hydrogen chloride, HCl, (Sigma-Aldrich, catalog number: H1758-100ML; CAS number: 7647-01-0), store at RT
30. Iron (II) sulfate heptahydrate, FeSO₄·7H₂O (Sigma-Aldrich, catalog number: F8263; CAS number: 7782-63-0), store at RT
31. Kanamycin sulphate monohydrate, C₁₈H₃₆N₄O₁₁·H₂SO₄·H₂O (Duchefa, catalog number: K0126; CAS number: 25389-94-0), store at 4 °C
32. Kinetin, C₁₀H₉N₅O (Sigma-Aldrich, catalog number: K3378; CAS number: 525-79-1), store at 4 °C

33. L-Arginine, H₂NC(=NH)NH(CH₂)₃CH(NH₂)CO₂H (Sigma-Aldrich, catalog number: A8094; CAS number: 74-79-3), store at RT
34. L-Aspartic acid, HO₂CCH₂CH(NH₂)CO₂H (Sigma-Aldrich, catalog number: A7219; CAS number: 56-84-8), store at RT
35. L-Glutamine, H₂NCOCH₂CH₂CH(NH₂)CO₂H (Sigma-Aldrich, catalog number: G8540; CAS number: 56-85-9), store at RT
36. Liquid nitrogen
37. L-Proline, C₅H₉NO₂ (Sigma-Aldrich, catalog number: P5607; CAS number: 147-85-3), store at RT
38. Magnesium chloride, MgCl₂ (Sigma-Aldrich; catalog number: M8266; CAS number: 7786-30-3), store at RT
39. Magnesium sulfate heptahydrate, MgSO₄·7H₂O (Sigma-Aldrich, catalog number: M2773; CAS number: 10034-99-8), store at RT
40. Maltose monohydrate, C₁₂H₂₂O₁₁·H₂O (Duchefa, catalog number: M0811; CAS number: 6363-53-7), store at RT
41. Manganese (II) sulfate monohydrate, MnSO₄·H₂O (Sigma-Aldrich, catalog number: M7899; CAS number: 10034-96-5), store at RT
42. Myo-Inositol, C₆H₁₂O₆ (Sigma-Aldrich, catalog number: I7508; CAS number: 87-89-8), store at RT
43. Nicotinic acid, C₆H₅NO₂ (Sigma-Aldrich, catalog number: N0761; CAS number: 59-67-6), store at RT
44. Potassium chloride, KCl (Sigma-Aldrich, catalog number: P5405; CAS number: 7447-40-7), store at RT
45. Potassium hydroxide, KOH (Fisher Scientific, catalog number: 10366240; CAS number: 1310-58-3), store at RT
46. Potassium iodide, KI (Sigma-Aldrich, catalog number: P8166; CAS number: 7681-11-0), store at RT
47. Potassium nitrate, KNO₃ (Sigma-Aldrich, catalog number: P8291; CAS number: 7757-79-1), store at RT
48. Potassium phosphate monobasic, KH₂PO₄ (Sigma-Aldrich, catalog number: P5655; CAS number: 7778-77-0), store at RT
49. Pyridoxine hydrochloride, C₈H₁₁NO₃·HCl (Sigma-Aldrich, catalog number: P6280; CAS number: 58-56-0), store at RT
50. Rifampicin, C₄₃H₅₈N₄O₁₂ (Sigma-Aldrich, catalog number: R3501; CAS number: 13292-46-1), store at 4 °C
51. Sodium chloride, NaCl (Sigma-Aldrich, catalog number: S7653; CAS number: 7647-14-5), store at RT
52. Sodium hydroxide, NaOH (Sigma-Aldrich, catalog number: 30620-1KG-M; CAS number: 1310-73-2), store at RT

53. Sodium molybdate dihydrate, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (Sigma-Aldrich, catalog number: 331058; CAS number: 10102-40-6), store at RT
54. Sodium phosphate monobasic, NaH_2PO_4 (Sigma-Aldrich, catalog number: S5011; CAS number: 7558-80-7), store at RT
55. Sucrose, $\text{C}_{12}\text{H}_{22}\text{O}_{11}$ (Duchefa, catalog number: S0809; CAS number: 57-50-1), store at RT
56. Thiamine hydrochloride, $\text{C}_{12}\text{H}_{17}\text{ClN}_4\text{OS} \cdot \text{HCl}$ (Sigma-Aldrich, catalog number: T1270; CAS number: 67-03-8), store at RT
57. Zinc sulfate heptahydrate, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (Sigma-Aldrich, catalog number: Z1001; CAS number: 7446-20-0), store at RT
58. FastAmp® Plant Direct PCR Kit (Intactgenomics, catalog number: 4612)
59. WTW™ TEP 4 Model Buffer Solution pH 4 (WTW™, catalog number: 108700)
60. WTW™ TEP 7 Model Buffer Solution pH 7 (WTW™, catalog number: 108702)
61. WTW™ TPL 10 Trace Model Buffer Solution pH 10.1 (WTW™, catalog number: 108805)
62. Rice soil composition (see Recipes)
63. Stock solution composition (see Recipes)
64. Phytohormones and antibiotics (see Recipes)
65. Cultivation medium composition (see Recipes)
66. Suspension medium (see Recipes)
67. Co-cultivation medium (see Recipes)
68. Resting medium (see Recipes)
69. Selection medium (see Recipes)
70. Pre-regeneration medium (see Recipes)
71. Regeneration medium (see Recipes)
72. Rooting medium (see Recipes)
73. Transformation cheat sheet (see Recipes)
74. GUS staining solution (see Recipes)
75. X-Gluc solution (see Recipes)

Equipment

Note: Equipment can be adapted to different lab conditions, however, PETRI DISHES, MAGENTA BOXES are VERY IMPORTANT TOOLS which affect transformation efficiency. We highly recommend to use the same brands as we used in this protocol. SCALPELS and TWEEZERS have to be high quality due to the frequent sterilization. Exact type and brand may be adapted to personal choice.

1. -80 °C Ultra low temperature freezer (Panasonic, model: MDF-U76V-PE)
2. Analytical and Precision Balances (Precisa Gravimetrics AG, Series 321LS, model: LS 2200C and LS 120A)

3. Autoclave (Systec, model: 3850 EL)
4. Benchtop pH meter; WTW™ inolab™ 7110 (Supplier: WTW™ 1AA114; Fisher Scientific, catalog number: 11731381)
5. Biospectrometer, basic (Eppendorf, catalog number: 6135000009)
6. Buckets, 5 L (Auer, catalog number: ER 5,6-226+DK)
7. Erlenmeyer flask, 250 ml (DURAN®, catalog number: 21 216 36)
8. Growth chamber 1 [Percival, model: CU-41L5; condition: 30 °C, continuous light (24/0 day/night photoperiod) with photosynthetic photon flux density (PPFD) of 200 µmol m⁻² s⁻¹ with PPFD-blue of 40 µmol m⁻² s⁻¹, PPFD-green of 80 µmol m⁻² s⁻¹ and PPFD-red of 70 µmol m⁻² s⁻¹]
9. Growth chamber 2 [Percival, model: CU-41L5; condition: 27 °C, 16/8 day/night photoperiod with photon flux density (PPFD) of 200 µmol m⁻² s⁻¹ with PPFD-blue of 30 µmol m⁻² s⁻¹, PPFD-green of 70 µmol m⁻² s⁻¹ and PPFD-red of 60 µmol m⁻² s⁻¹]
10. Glass bead sterilizer (SkinMate Apus Quartz)
11. High precision tweezers (110 mm; type 5.SA; extra fine tip; stainless steel) (Behr Labor-Technik, catalog number: 6.266 876)
12. Electroporator (Bio-Rad Gene Pulser™)
13. Incubator/shaker 28 °C (Infors HT, Multitron Standard)
14. Inoculating loops, PS 10UL PK/25 (Hach, catalog number: 2749125)
15. Laboratory bottles with screw cap and pouring ring, 100 ml (Duran®, catalog number: 218012458)
16. Laboratory bottles with screw cap and pouring ring, 250 ml (Duran®, catalog number: 218013651)
17. Laboratory bottles with screw cap and pouring ring, 500 ml (Duran®, catalog number: 218014459)
18. Laboratory bottles with screw cap and pouring ring, 1,000 ml (Duran®, catalog number: 218015455)
19. Lightmeter (Quantum Spectrometer, UPRtek, PAR 300)
20. Magnetic stirring bars, octagonal, with pivot ring, blue, 38 mm (VWR, catalog number: 442-0438)
21. Pipette controller, Pipetboy acu 2 (Supplier: Integra (Biosciences); for glass and plastic pipettes from 0.1 to 100 ml) (VWR, catalog number: 613-4437)
22. Polycarbonate vacuum desiccator (Sanplatec, catalog number: PC-250KG)
23. Pots SM-H Container 2.0 L (Meyer-shop, catalog number: 737203)
24. Scalpel handles (Supplier: BAYHA GMBH; length: 160 mm) (VWR, catalog number: 233-5202)
25. Stereomicroscope (Zeiss, Discovery.v8, brightfield images)
26. Stereo zoom and fluorescence microscope (Zeiss, AxioZoom.V16) for GFP imaging
27. Sterile bench (Thermo Scientific™ Heraguard™ ECO Clean Bench)
28. Thermometer (Laserliner ThermoSpot)
29. Thermal cycler (Bio-Rad, model: T100™, catalog number: 1861096)
30. Vacuum pump (Vacuubrand, catalog number: MZ 2C)

Procedure

E. Prepare stock solutions

- a) Prepare 17 stock solutions including N6 major 1, N6 major 2, N6 major 3, N6 major 4, B5 minor 1, B5 minor 2, B5 minor 3, B5 minor 4, B5 vitamins, AA macro salts, AA micro salts, Glycine, MS 1, MS 2, MS 3, MS 4 and MS vitamins with composition according to [Table S2](#). Prepare phytohormone and antibiotic stock solutions according to [Table S3](#).

- b) Filter sterilize all stock solutions and keep at 4 °C.

Notes:

- a. Always filter sterilize the stock solutions (filter pore size: 0.2 µm, **Consumables** 1, 2, 3, 20 and 21 for filter types), do not autoclave!
- b. Stock solutions can be stored at 4 °C for a maximum of 3 months.
- c. Do not use stock solutions older than 3 months.

F. Prepare cultivation medium

- a) Prepare cultivation medium according to [Table S4-Table S11](#). Cultivation medium composition.

Notes:

- a. It is very critical to adjust pH properly: always calibrate the pH meter before use (for pH 5.8, use calibration solutions pH 4 and pH 7).
- b. Always adjust the pH very precisely: for a medium which e.g., pH 5.8 is needed, accepted pH value is 5.80-5.81.
- c. Record the pH values before and after pH adjustment.
- d. Always measure the pH at the same medium temperature and record the medium temperature while measuring pH (Figure 3).

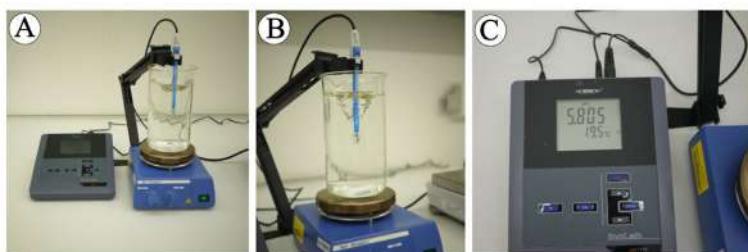


Figure 3. pH measuring. A. Recording pH and temperature while preparing medium. B. Detail of the right position of the electrode while recording the pH. C. Temperature and pH recording at the display.

- e. pH is measured after all components are added, except for agarose, phytohormones and antibiotics (see [Table S4-Table S11](#) for more details), before autoclaving the medium.
- f. It is critical to use short autoclave cycles for autoclaving media: apply a sterilization time of 5 min (!) at 121 °C (101 kPa) and transfer media to RT as soon as the autoclave has cooled

down to 95 °C, do not let media stay longer in the autoclave (Figure 4). Sterilization time varies depending on the amount of medium because the autoclaving process includes additional time for the heating up and cooling down. In this protocol, we prepared medium in 0.5 L bottles, and applied a sterilization time of 5 min at 121 °C (101 kPa), the whole autoclave cycle took 1.5 h.

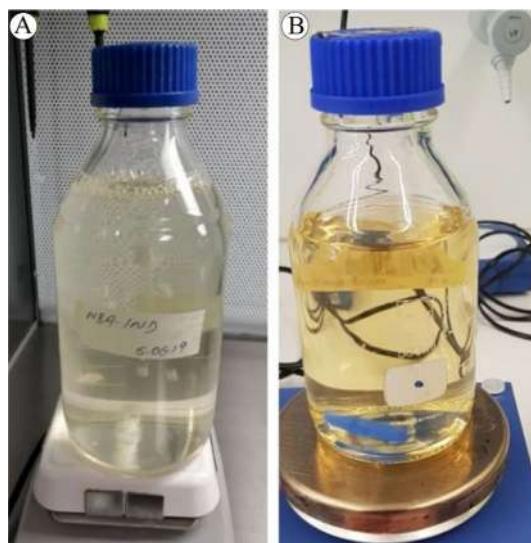


Figure 4. Comparison of media autoclaved for 5 min (A) and 20 min (B) at 121 °C (101 kPa). The caramel color is a good indication of over-autoclaved media. Media color should remain almost colorless after autoclaving like show on the left picture.

- g. Add phytohormones and antibiotics after autoclaving only when media have cooled to 40–45 °C (always use the Laserliner thermometer to check temperature!).
- h. Pour plates under sterile bench (biosafety cabinet), see Table S4 for types of Petri dishes and amount to pour for each medium. Types of Petri dishes are different for different media and it is critical to follow our recommendation.
- i. Close the lids only when plates have completely dried and no water condensation is visible on the side of Petri dishes (Figure 5). This is critical because calli prefer to stay dry on the medium, wet calli do not regenerate. But also, do not overdry. Overdried media shrink and form cracks, and due to reduced water content, solute concentrations increase substantially, affecting the sensitive calli and the regeneration process.
- j. Plates are wrapped with stretch foil and stored at room temperature and in the dark (Figure 5). Do not refrigerate media.
- k. Discard unused plates with solid media after 2 weeks.

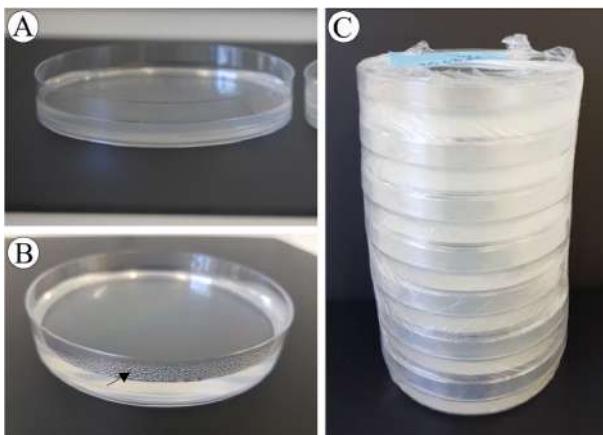


Figure 5. Medium drying and storage. A. Completely dried Petri dish. B. Wet Petri dish, with condensed water; the black arrow indicates condense water. C. Plates are wrapped and stored at RT and in the dark.

G. Competent *Agrobacteria* preparation and transformation

- a) Prepare *Agrobacterium* (LBA4404 or EHA105) competent cells.
 - i. Streak out *Agrobacterium* cells from 10% glycerol stock (stored at -80 °C) on a plate with solid YEP medium containing 20 mg/L rifampicin and incubate for 2 days in the dark at 28 °C.
 - ii. Pick a single colony with a sterile tooth pick or pipette tip and culture in a glass test tube with 5 ml YEP liquid medium containing 20 mg/L rifampicin overnight (12 to 16 h) in the dark and at 28 °C and shake at 200 rpm.
 - iii. Sub-culture 1 ml overnight culture into 35 ml liquid YEP medium plus 20 mg/L rifampicin in a 250 ml flask, incubate at 28 °C, dark and shake at 200 rpm for about 8 h until OD₆₀₀ reaches 0.5-0.8.
 - iv. Chill *Agrobacteria* on ice for 5 min.
 - v. Centrifuge the bacteria in a 50 ml Falcon® tube at 3,000 x g, 4 °C for 5 min.
 - vi. Resuspend the pellet in 1 ml 20 mM CaCl₂ in a 50 ml Falcon® tube (for 50 ml YEP, add 1 ml 1 M CaCl₂).
 - vii. Aliquot (50 µl) in a 2 ml safe lock microcentrifuge tube, flash freeze with liquid nitrogen and store at -80 °C.
- b) Transformation of competent *Agrobacterium* cells (LBA4404 or EHA105) with GUS/GFP reporter constructs.
 - i. Take an aliquot (50 µl) of competent *A. tumefaciens* LBA4404 or EHA105 from 10% glycerol stock (stored at -80 °C) and thaw on ice for 10 min.
 - ii. Mix cells with 1 µl (~100 ng) plasmid DNA in a 1.5 ml microcentrifuge tube.
 - iii. Fill mixture into a sterile ice-cold electroporation cuvette (0.2 cm gap).
 - iv. Perform electroporation using an electroporator at 1.8 kV. Pressing until ‘buzzing’ sound can be heard (about 5 ms), leave all other settings as default: 200 Ω, capacitance extender 250 µFD, capacitance 25 µFD.
 - v. Add 1 ml YEP medium without antibiotics immediately after pulse, mix cells by pipetting up

- and down using 1 ml pipet tip.
- vi. Transfer suspension into an autoclaved 1.5 ml microcentrifuge tube and incubate for 30 min at 28 °C without shaking.
 - vii. Spin cells down for 1 min at 210 x g.
 - viii. Carefully remove supernatant, but leave about 100 µl medium in the 1.5 ml microcentrifuge tube.
 - ix. Re-suspend the cells in the remaining medium in a 1.5 ml microcentrifuge tube and place 100 µl on a YEP agar plate containing 50 mg/L kanamycin and 20 mg/L rifampicin.
 - x. Incubate the plates for 2 days at 28 °C in the dark.
 - xi. Conduct colony PCR to select for transformed colonies by checking for the presence of the selection marker gene. In our case, to confirm the presence of the *Hpt* gene (hypoxanthine phosphoribosyl transferase encoding gene), we used the following primers: *Hpt_F*: 5'-AGCCTGACCTATTGCATCT-3'; *Hpt_R*: 5'-CATATGAAATCACGCCATGT-3', amplicon size 200 bp, T_m 55 °C.
 - xii. Select 1-3 positive colonies and inoculate in a glass test tube with 3 ml liquid YEP medium containing 50 mg/L kanamycin and 20 mg/L rifampicin. Grow bacteria at 28 °C in the dark with shaking speed at 200 rpm overnight.
 - xiii. Prepare glycerol stock of *Hpt* positive *Agrobacterium* cultures by mixing 0.6 ml culture with 0.3 ml 10% glycerol (autoclaved) in 2 ml safe lock microcentrifuge tubes, freeze immediately in liquid N₂ and store at -80 °C.

Note: In this study we used a GUS reporter construct (pSWEET13:GUSplus) and a GFP reporter construct (pOsUbi:eGFP) (Figure 6) which were kindly provided by Dr. Joon-Seob Eom (Heinrich Heine University, Düsseldorf, Kyung Hee University, South Korea) and Dr. Bing Yang (University of Missouri, USA) respectively. From other experiments, we know that under pSWEET13 and OsUbi promoters, GUS and GFP are expressed at both calli and seedling stages, therefore we used these two constructs for checking transformation efficiency. One can use any other GUS/GFP reporter construct for the same purpose. Optimal is the use of GUS or GFP intron constructs, since Agrobacteria do not splice the intron and thus can not cause false positive calli or regenerate plants (Vancanneyt et al., 1990).

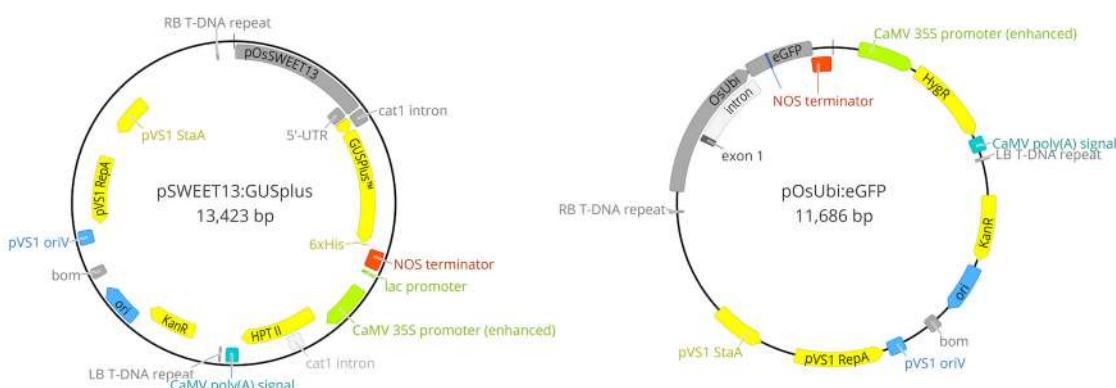


Figure 6. Maps of the two plasmids carrying GUS and GFP reporter genes used in this study

H. Isolation of the immature embryos

14. Harvest rice immature seeds (8-12 days post-pollination) and place 300-400 seeds in a 50 ml Falcon® tube. It is important to get immature seeds at the right developmental stage. To check for the stage of immature embryos, squeeze immature seeds gently and feel their hardness. Immature seeds should be at the late milky stage at which the endosperm is not completely cellularized yet, see Video 1 for more details. This harvesting step can be done outside of a sterile hood, but all steps below including dehusking, sterilization, isolation of immature embryos are done under sterile condition.



Video 1. Selection of immature seeds

15. Under sterile condition, wash seeds with 40 ml ethanol 70% for 30 s with hand shaking in a 50 ml Falcon® tube; discard ethanol. Then wash seeds with 40 ml sterile Milli-Q water three times, discard the water.
16. Place immature seeds into a Petri dish layered with moistened sterile filter papers and remove the seed coat (lemma and palea) very carefully under a stereo microscope and in sterile conditions (under laminar flow chamber) with scalpel and forceps (Video 2, Figures 7A-7B).

Scalpel and forceps should be sterilized by using for example a glass bead sterilizer. Tools need to be cooled down to avoid heat damage to immature embryos.



Video 2. Dehusking of immature seeds

17. Place the immature seeds in sterile conditions in a 50 ml Falcon® tube (once lemma and palea have been removed) to start the sterilization procedure (Figure 7C).
18. Add 40 ml ethanol 70%, under sterile conditions, to the Falcon® tube and shake by hand for 30 s. Discard ethanol. Wash immature seeds with 40 ml sterile Milli-Q water. Discard the water.
19. In a sterile 50 ml Falcon® tube, prepare a mixture of 5 ml commercial bleach Klorix® with 35 ml sterile Milli-Q water [final concentration of NaClO is 28 g/L (0.376 M)].
20. Add the mixture to the 50 ml Falcon® tube containing the immature seeds and shake by hand for 5 min.
21. Discard the Klorix® mixture and rinse the seeds with 40 ml sterile Milli-Q water, repeat the procedure 15 times until all remains of Klorix® are completely washed out.
Note: It is very important that Klorix® is washed out completely, because Klorix® can affect the germination of immature embryos. We recommend to rinse the immature embryos in a 50 ml Falcon® tube 15 times with 40 ml sterile Milli-Q water each time.
22. Place immature seeds into a Petri dish with sterile filter paper and carefully isolate immature embryos (IEs) using scalpel and fine forceps under the stereo microscope (Video 3, Figure 7D) in a sterile hood. Be careful and gentle to avoid damaging or wounding the immature embryos!



Video 3. Immature embryo isolation

23. Only select undamaged immature embryos with sizes between 1.3-1.8 mm. The immature embryos should be opaque, off-white colored. Transparent immature embryos will not germinate. Place about 50 immature embryos (scutellum face up) on co-cultivation medium (Figure 7E).

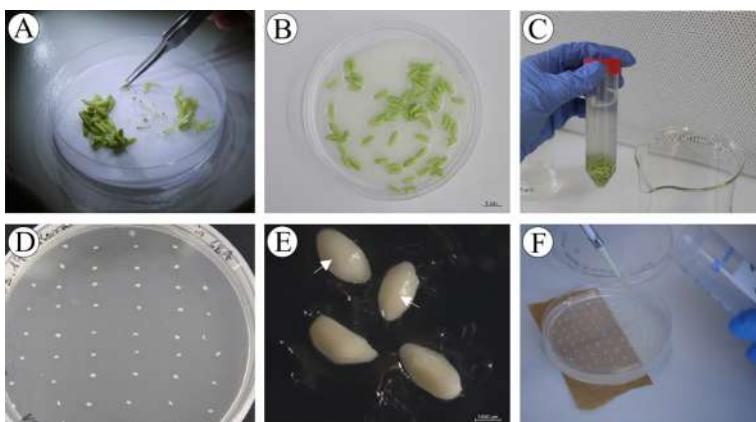


Figure 7. Immature embryo isolation and inoculation with *Agrobacteria*. A. Removal of palea and lemma under a stereo microscope. B. Dehusked immature seeds in Petri dish with wet Whatman® paper. Scale bar: 1 cm. C. Seed sterilization with ethanol and Klorix®. D. Isolated immature embryos in a plate. E. Immature embryos with scutellum up (white arrows) and immature embryos lying on the side. Scale bar: 1 mm. F. Inoculation with *Agrobacteria*.

I. Transformation and co-cultivation

Notes:

1. Generally, we recommend to start with 100 immature embryos for one transformation. Depending on the transformation efficiency, the number of immature embryos as starting materials can be reduced or increased.
2. We recommend to use 12 immature embryos as controls without Agrobacterium inoculation. In the first selection step, 6 control immature embryos will be moved onto selection medium without hygromycin B to check for the regeneration ability. The other 6 control immature embryos will

be moved onto selection medium with 30 mg/L hygromycin B to control the effectiveness of hygromycin B selection. These are very important controls, especially when trying to adapt this transformation protocol to other varieties. Hygromycin B concentration, regeneration medium and inoculation time may have to be adapted for other varieties.

3. We also recommend to record all data for each transformation, e.g., date, number of immature embryo as well as any notes (see [Table S12](#) for the template).

1. Strike *Agrobacteria* (LBA4404 or EHA105) from frozen -80 °C stocks on solid YEP plates containing antibiotics (50 mg/L kanamycin and 20 mg/L rifampicin) two days before infection. Then incubate for two days at 28 °C in the dark.
2. On the day of the transformation, take a 3 mm-size loop of *Agrobacterium* culture from the YEP plate and suspend in suspension medium (Table S5) in a 50 ml Falcon® tube.
3. Vortex bacterial suspension and adjust to OD₆₀₀ 0.3. Incubate in dark at 25 °C for 1 h, no shaking needed.

Notes:

- a. Add acetosyringone just before use and prepare freshly!
 - b. Dissolve acetosyringone: for 50 ml suspension culture, dissolve 10 mg acetosyringone in 100 µl DMSO and then take 9.81 µl solution to obtain 0.981 mg acetosyringone; no filter sterilization needed.
 - c. Always adjust OD₆₀₀ very precisely: recommended values, depending on the spectrometer, are between 0.30 and 0.31.
4. Drop 5 µl *Agrobacterium* suspension on top of each immature embryo, with scutellum side-up (Figure 7F).
 5. Incubate plates with embryos in the presence of *Agrobacterium* for 7 days at 25 °C in the dark (Figure 8).

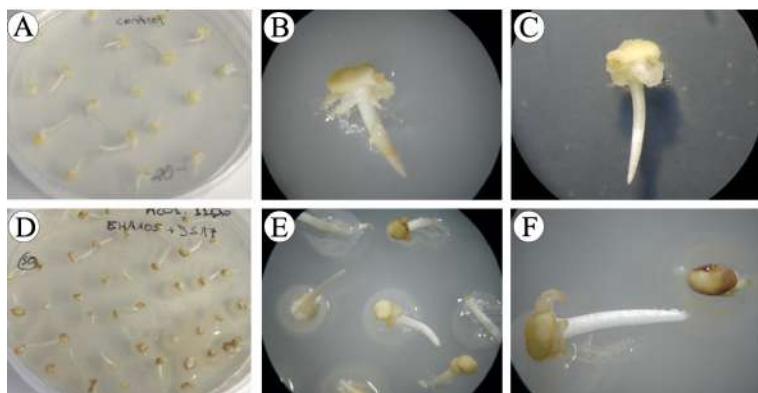


Figure 8. Immature embryos at the end of co-cultivation phase. A-C. Control calli without *Agrobacterium* infection. D-F. *Agrobacterium*-infected calli.

J. Resting

1. Check immature embryos for possible contamination (Figure 9). If immature embryos are contaminated, discard them. If possible, uncontaminated immature embryos in the contaminated plate can be rescued and placed on a separate plate. Otherwise discard the whole plate. Contamination can happen if dehusking or immature embryos isolation was not done carefully enough and embryos were damaged with forceps or scalpel during the process, facilitating contamination with other microorganisms (Figure 9).

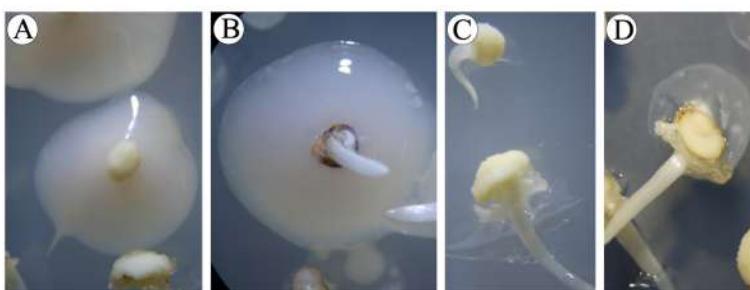


Figure 9. Contamination of immature embryos. A. Control immature embryos (no *Agrobacterium* inoculation) showing bacterial contamination. B. *Agrobacterium*-inoculated immature embryos showing contamination with other bacteria. C. Control non-infected immature embryos without contamination. D. *Agrobacterium*-inoculated immature embryos without contamination.

2. Place immature embryos without contamination on sterile filter paper and remove shoots with scalpel and forceps. Performing this steps under a stereo-microscope to guarantee complete excision of shoots.
 - a. No parts of the shoot and rim should be left over, remove everything (Figures 10A and 10B).
 - b. Remove the brown tissue from calli if there is excess, but be careful not to damage the immature embryos.

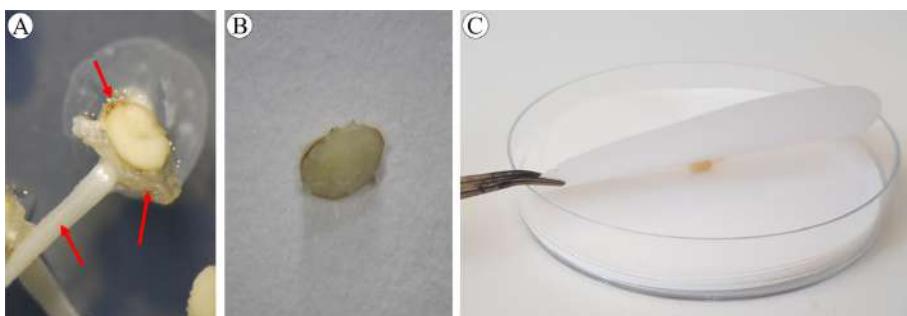


Figure 10. Immature embryos before (A) and after (B) removing shoots. Arrows indicate parts to be removed: shoot (bottom left), rim (bottom right) and brown tissue (upper). C. Cleaning immature embryo using two layers of sterile filter paper.

3. Clean the immature embryos by placing them between two layers of sterile filter paper (Figure 10C) and carefully dab off the *Agrobacteria*. Repeat this procedure at least two times to remove the surplus *Agrobacteria*.
4. Transfer immature embryos scutellum side-up onto resting medium (16 immature embryos/plate) and incubate for 5 days in growth chamber 1 [30 °C, continuous light, 24/0 day/night photoperiod, with photosynthetic photon flux density (PPFD) of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with PPFD-blue of 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$, PPFD-green of 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and PPFD-red of 70 $\mu\text{mol m}^{-2} \text{s}^{-1}$] (Figure 11).

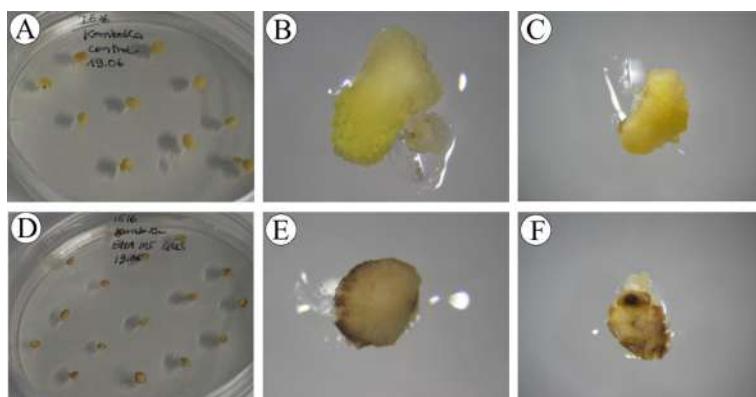


Figure 11. Immature embryos at the end of the resting phase. A-C. Control immature embryos. D-F. *Agrobacterium*-inoculated immature embryos.

Notes:

- a. Use the stereo microscope to check for the state of the immature embryos (e.g., if there is any contamination), to clean brown tissues and take immature embryos more gently.
- b. Do not push immature embryos into the medium, let them stay loosely on top of the medium.
- c. Keep immature embryos on the same position when transferring between media (scutellum up-side to the medium).
- d. Seal plates with two layers of Micropore 3M tape.

K. Selection

1. After 5 days of resting, remove the brown tissue completely with forceps and a scalpel by scratching or cutting brown tissue off the surface of the immature embryo. Transfer the immature embryos onto the selection medium, containing 30 mg/L hygromycin B (16 calli/plate). Incubate at 30 °C for 10 days in continuous light [24/0 day/night photoperiod, photosynthetic photon flux density (PPFD) of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with PPFD-blue of 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$, PPFD-green of 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and PPFD-red of 70 $\mu\text{mol m}^{-2} \text{s}^{-1}$] (1st selection) (Figure 12).

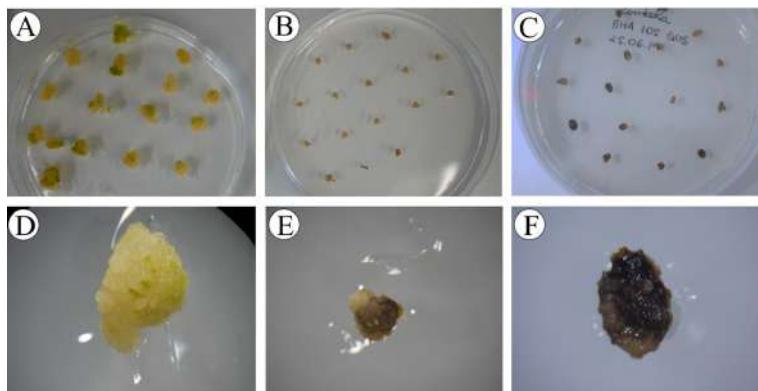


Figure 12. Immature embryos at the end of the first selection phase. A, D. Control immature embryos without hygromycin B, immature embryos produced compact and embryogenic calli and started turning green. B, E. Control immature embryos on selection medium with 30 mg/L hygromycin B. Immature embryos do not turn brown completely but are also not growing either. C, F. *Agrobacterium*-inoculated immature embryos may turn brown partially or completely.

2. After 10 days of 1st selection, transfer the calli to a freshly prepared plate with selection medium (16 immature embryos/plate). Remove brown tissue as much as possible from the immature embryos and incubate at 30 °C for 10 days in continuous light [24/0 day/night photoperiod, photosynthetic photon flux density (PPFD) of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with PPFD-blue of 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$, PPFD-green of 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and PPFD-red of 70 $\mu\text{mol m}^{-2} \text{s}^{-1}$] (2nd selection) (Figure 13). Some immature embryos turn brown completely after the 1st selection. In this case, do not remove the brown tissue and keep the whole immature embryos.

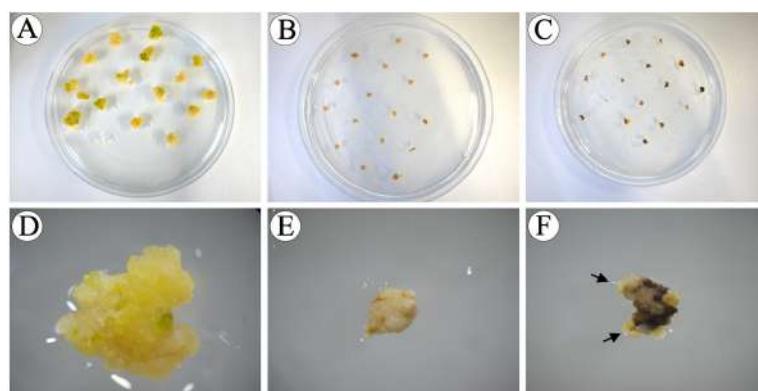


Figure 13. Immature embryos after the 2nd selection phase. A, D. Control immature embryos without hygromycin B produced proliferating embryogenic calli. B, E. Control immature embryos with 30 mg/L hygromycin B, immature embryos remain their size (2-4 mm) without producing any callus. C, F. *Agrobacterium*-inoculated immature embryos on selection medium produced small, compact and light yellow embryogenic microcalli (black arrows).

3. After 10 days of 2nd selection, take only the embryogenic microcalli from the black immature embryos and place onto fresh selection medium (16 immature embryos/plate), incubate at 30 °C

for 10 days in continuous light [24/0 day/night photoperiod, photosynthetic photon flux density (PPFD) of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with PPFD-blue of 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$, PPFD-green of 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and PPFD-red of 70 $\mu\text{mol m}^{-2} \text{s}^{-1}$] (3rd selection) (Figure 14). The embryogenic calli are small, compact clusters of cells, usually light yellow in color (Figure 13 F). Non-embryogenic calli are usually larger, soft, semi-transparent and yellow or gray loosely- held clusters of cells. The “mother immature embryo” can be kept on the selection medium as a back-up in case the freshly-isolated microcalli do not grow, or more microcalli are needed. For this step, microcalli from different immature embryos are kept separately by dividing the Petri dish into small areas (Figure 14 C). The separated microcalli will be considered as independent events.

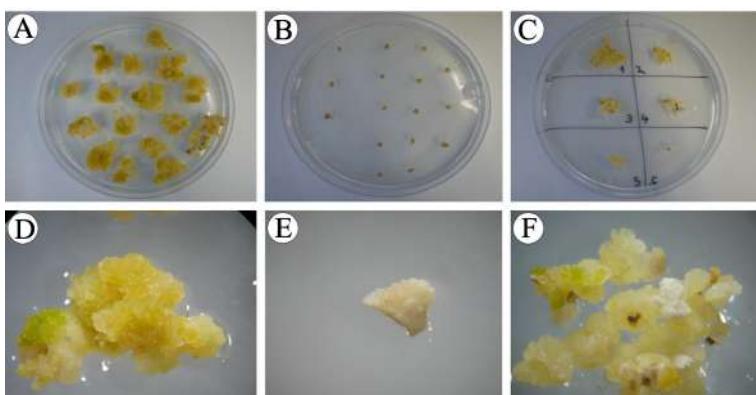


Figure 14. Microcalli at the end of the 3rd selection phase. A, D. Control calli without hygromycin B are compact, embryogenic and turned green partly. B, E. Control immature embryos with 30 mg/L hygromycin B, immature embryos remain the same size and do not grow. C, F. *Agrobacterium*-inoculated microcalli proliferating on selection medium.

Notes:

- a. Use the stereo microscope to check for the state of the immature embryos/calli, to remove the brown tissue and to select the embryogenic calli more precisely.
- b. Don't push immature embryos/calli into to the medium, let them stay loosely on top of the medium.
- c. Seal plates with two layers of Micropore 3M tape.
- d. If no microcalli is generated after 2nd selection, refresh the selection medium and culture for another 10 days. If after 2-3 additional rounds of selection, no microcalli are produced, discard the immature embryos.

L. Plant regeneration

1. Transfer resistant calli to pre-regeneration medium containing 30 mg/L hygromycin B (6-9 calli/plate) and incubate at 30 °C for 10 days in continuous light [24/0 day/night photoperiod, photosynthetic photon flux density (PPFD) of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with PPFD-blue of 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$, PPFD-green of 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and PPFD-red of 70 $\mu\text{mol m}^{-2} \text{s}^{-1}$] (Figure 15).

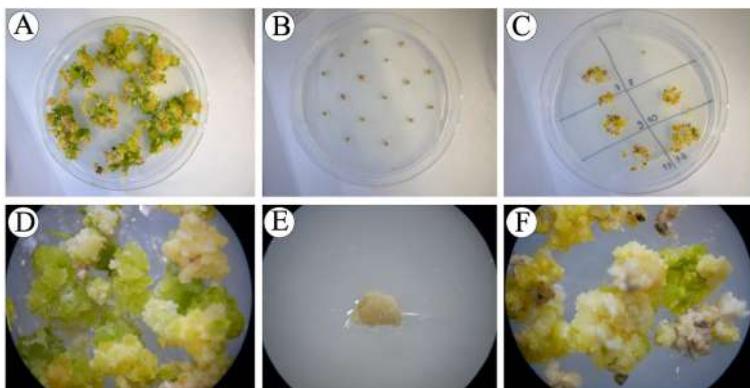


Figure 15. Calli at the end of pre-regeneration phase. A, D. Control calli without hygromycin B are mostly green. B, E. Control immature embryos with 30 mg/L hygromycin B; immature embryos remain their sizes and did not grow. C, F. *Agrobacterium*-inoculated proliferated calli with greening spots.

2. Select proliferating calli with green spots that covered approximately 2/3 of the calli (Figure 15F) and transfer to regeneration medium containing 30 mg/L hygromycin B (6 calli/plate), for shoot development and incubate at 30 °C for 14 days in continuous light [24/0 day/night photoperiod, photosynthetic photon flux density (PPFD) of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with PPFD-blue of 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$, PPFD-green of 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and PPFD-red of 70 $\mu\text{mol m}^{-2} \text{s}^{-1}$] (Figure 16).

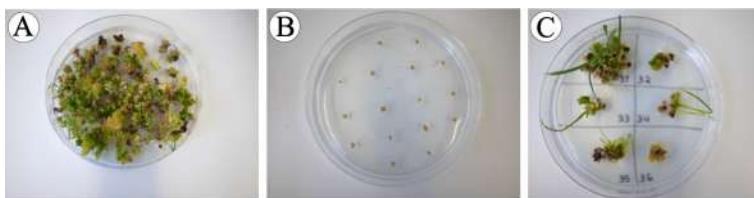


Figure 16. Calli at the end of the Regeneration phase. A. Regenerated plants from a control calli without hygromycin B. B. Control immature embryos with 30 mg/L hygromycin B, immature embryos did not grow. C. Regenerated plants from *Agrobacterium*-inoculated calli are putative transformants.

3. If calli have not produced any shoot or only produce small shoots, transfer the calli to freshly prepared plates of regeneration medium (6 calli/plate) every 14 days and incubate at 30 °C in continuous light [24/0 day/night photoperiod, photosynthetic photon flux density (PPFD) of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with PPFD-blue of 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$, PPFD-green of 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and PPFD-red of 70 $\mu\text{mol m}^{-2} \text{s}^{-1}$], until small plantlets develop (small rice plants regenerated from a callus with at least two leaves and small root system, Figures 16–17).
4. Select 1–3 plantlets from each callus and transfer into a Magenta™ GA-7 vessel containing rooting medium without hygromycin B. Incubate them for 7 days at 27 °C [16/8 day/night photoperiod with photon flux density (PPFD) of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with PPFD-blue of 30 $\mu\text{mol m}^{-2} \text{s}^{-1}$, PPFD-green of 70 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and PPFD-red of 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$]. At this stage, plantlets

regenerated from different calli are considered independent transformation events, therefore taking more than one plantlet as a backup to ensure that all putative events grow vigorously in the rooting step.

5. After 7 days, when plantlets reach the top of the Magenta™ box, place another Magenta™ box on top to create more space for the plants to grow (Figure 17A). Place ‘double’ Magenta™ box in the growth chamber with 27 °C [16/8 day/night photoperiod with photon flux density (PPFD) of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with PPFD-blue of 30 $\mu\text{mol m}^{-2} \text{s}^{-1}$, PPFD-green of 70 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and PPFD-red of 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$] for another 7 days.
6. Transfer plantlets of approx. 15 cm height into soil by gently washing away excess agarose attached to roots. Grow plantlets in a pot which is submerged in a larger bucket to maintain high humidity condition in the glass house with natural daylight and additional lamplight of 8/16 day/night photoperiod with a day temperature of 30 °C ± 2 °C and a night temperature of 25 °C ± 2 °C. The relative humidity was between 50-70%. Plants were grown under a photosynthetic active radiation (PAR) or photosynthetic photon flux density (PPFD) of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with PPFD-blue of 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$, PPFD-green of 70 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and PPFD-red of 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

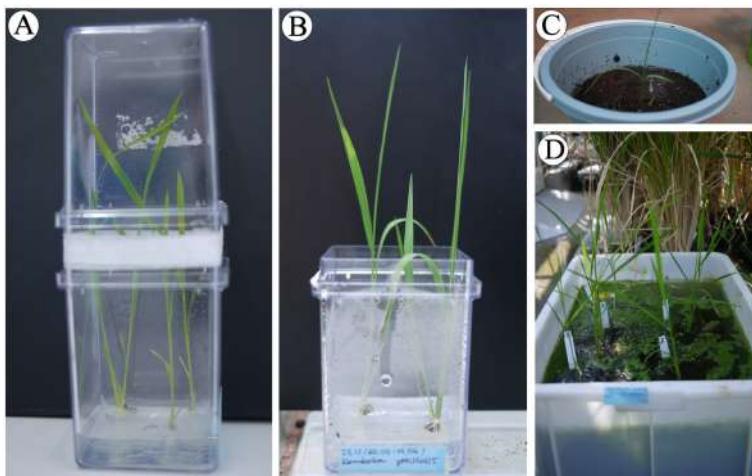


Figure 17. Rooting and planting. A, B. Two-week-old regenerated plants, before transfer to soil. C. Regenerated plant right after planting. D. Regenerated plants, two weeks after planting.

Notes:

- a. Use the stereo microscope to check for the state of the calli/plantlets, to select the calli and separate plantlets more gently.
- b. Don't push the calli into to the medium, let them stay loosely on top of the medium.
- c. Seal Petri dishes or Magenta™ GA-7 vessels with two layers of Micropore 3M tape.
- d. Since the plantlets were regenerated under continuous light (24/0 day/night photoperiod), we recommend to grow the plantlet for the rooting step under long-day conditions (16/8 day/night photoperiod) to slowly acclimate them to the upcoming short-day conditions in the glasshouse (8/16 day/night photoperiod).

M. Screening for transformed plants

Note: In this protocol, we did not unambiguously determine transformation events. We used plants that had been regenerated in parallel, but without infection by Agrobacteria as controls. In these control plants, we did not observe GUS staining or GFP fluorescence. All transformation events counted here were derived from independent immature embryos (one plant per one transformed immature embryo).

- a) Transformation of 100 Komboka immature embryos with GUS-intron reporter construct using two different *Agrobacterium* strains (EHA105 and LBA4404) resulted in 14 and 31 putative transformants from independent immature embryos, respectively (Table 1). All regenerated plants were tested for GUS activity. All leaves of regenerated plants showed GUS activity (Figures 18A-18B), except control plants which also underwent the whole protocol but without *Agrobacterium* infection (Figure 18C). Our results might indicate that the LBA4404 strain is more efficient in transforming Komboka when compared to EHA105, however the relative efficiency of different *Agrobacterium* strains can not be judged without careful quantitative analyses across many independent transformations.

Table 1. Apparent transformation efficiency of Komboka using different *Agrobacterium* strain

Construct/ <i>Agrobacterium</i> strain	# immature embryos	# putative independent events	# GUS positive events
GUS/LBA4404	100	31	31
GUS/EHA105	100	14	14

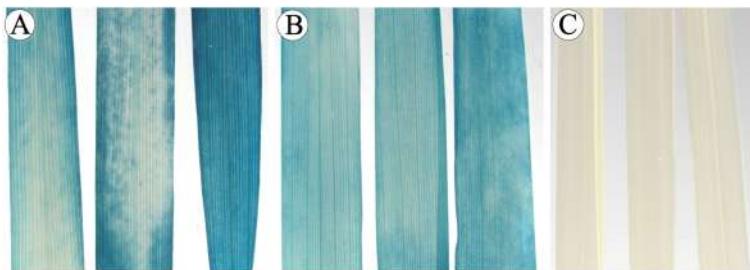


Figure 18. GUS histochemistry of transformed Komboka leaves. A, B. GUS-stained leaves of two plants which are regenerated from two independent immature embryos. C. GUS-stained leaves from one control plant regenerated without *Agrobacterium* infection, no GUS activity detectable.

- b) Transformation of Komboka with a GFP reporter construct using the *Agrobacterium* strain LBA4404 resulted in 54 putative independent events (Table 2). Roots of all these 54 plants were GFP positive (Figures 19A-19E). No GFP fluorescence was observed in uninfected plants (Figure 19F). From 54 GFP positive events, 200 bp GFP gene fragment was amplified by PCR

from 48 plants (Figure 20). Full T-DNA insertion, inheritance and copy number remain to be validated.

Table 2. Apparent transformation efficiency of Komboka with GFP reporter construct

Construct/ <i>Agrobacterium</i> strain	# immature embryos	# putative independent events	# GFP positive events	# PCR positive events
GFP/LBA4404	100	54	54	48

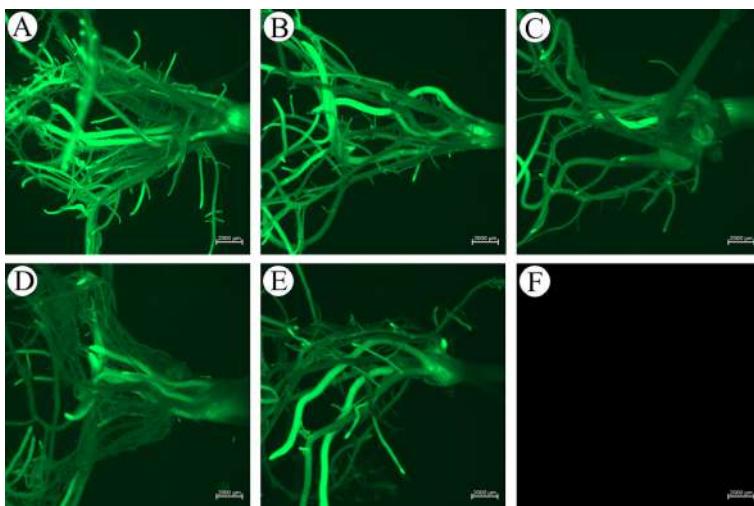


Figure 19. GFP fluorescence of transformed Komboka. A-E. Root of five independent transformants under blue light. F. Root of a non-infected plant regenerated from tissue culture (control). Scale bar: 2000 μ m. All photos are taken and displayed at the same setting.

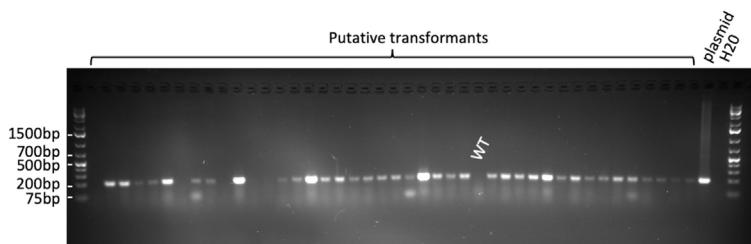


Figure 20. Representative gel picture for confirmation of a 200 bp GFP gene fragment in transformed plants by PCR. WT: non-infected plant regenerated from tissue culture.

N. Adjusting the protocol for transformation of other rice varieties

To adapt this protocol for the transformation of other varieties, we recommend to evaluate the efficiency after each step using GUS or GFP intron reporter constructs (Vancanneyt *et al.*, 1990). For example, after co-cultivation, use five calli for GUS staining or GFP screening to test whether the co-cultivation step was successful or whether co-cultivation time needs to be adjusted.

We also recommend to use non-infected immature embryos as controls to check for the

effectiveness of hygromycin B selection as well as the regeneration ability on media without hygromycin B (see Note 2, Section E), because hygromycin B sensitivity and regeneration ability may differ among varieties. Hygromycin B concentrations can be adjusted between 5-50 mg/L. Criteria for adjustment include: control immature embryos cannot grow or produce microcalli on hygromycin B containing media (Figures 13, 14, 15).

Another relevant parameter is the stage of immature embryo development, which may differ between varieties; immature seeds should be in the late milky stage (Video 1), typically with a size of 1.3-1.8 mm at about 8-12 days post pollination. *Agrobacterium* strains (AGL1, LBA4404, EHA105) can be compared. Also, the number of rounds of selection and rounds of pre-generation steps can be adjusted between 1-3 until microcalli or green spots are produced.

Data analysis

A. GUS staining

1. Harvest 3 cm leaves and place in 15 ml Falcon® tubes.
2. Add 5 ml staining solution (Tables S13 and S14).
3. Vacuum the samples for 10 min at RT.
4. Close the tubes and incubate at 37 °C overnight in the dark.
5. Remove staining solution.
6. Add 70% ethanol for destaining (chlorophyll removal) and incubate samples at 65 °C. The duration of incubation affects destaining, the longer the tissue will be incubated, the higher contrast of blue dye to background can be achieved, usually 2-3 days are fine. During this incubation time, exchange 70% ethanol for 1 or 2 times
7. As long as the tissue stays in ethanol, it can be stored at RT for long time (at least several months without losing the color). It is recommended to store the samples in a dark place.

B. GFP imaging

Roots of GFP-transformed Komboka were observed under blue light with a Zeiss AxioZoom.V16 stereo microscope, filter excitation wavelength: 450-490 nm, filter emission wavelength: 500-550 nm, excitation wavelength: 488 nm, emission wavelength: 509 nm, exposure time 4.59 s, zoom: 0.7, total optical magnification: 7x. All photos are taken and displayed at the same setting: black: 2000, gamma: 1.0, white: 22897.

C. PCR for GFP encoding gene

To check for the presence of the GFP gene in regenerated plants, FastAmp® Plant Direct PCR Kit (Intactgenomics) was used to amplify a 200 bp fragment of the GFP coding region using the following primers: VL_GFP_F1 5'-GCAAGCTGACCCTGAAGTTC-3', VL_GFP_R1 5'-GTCTTAGTTGCCGTCGTC-3'. PCR conditions: initial denaturation step at 95 °C for 5 min, followed by 35 cycles each at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 20 s, last extension at

72 °C for 10 min and the reaction was kept at 10 °C.

Recipes

Note: See [Supplementary file \(Tables S1-S14\)](#) for Recipes.

1. Rice soil composition (Table S1)
2. Stock solution composition (Table S2)
3. Phytohormones and antibiotics (Table S3)
4. Cultivation medium composition (Table S4)
5. Suspension medium (Table S5)
6. Co-cultivation medium (Table S6)
7. Resting medium (Table S7)
8. Selection medium (Table S8)
9. Pre-regeneration medium (Table S9)
10. Regeneration medium (Table S10)
11. Rooting medium (Table S11)
12. Transformation cheat sheet (Table S12)
13. GUS staining solution (Table S13)
14. X-Gluc solution (Table S14)

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Competing interests

The Institute for Molecular Physiology, Heinrich Heine University of Düsseldorf (HHU), Germany, The Center for Tropical Agriculture (CIAT), Cali, Colombia and the International Rice Research Institute (IRRI), Philippine contributed equally towards the development of the protocol. The authors declare no competing interests.

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Multitarget Immunohistochemistry for Confocal and Super-resolution Imaging of Plant Cell Wall Polysaccharides

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[Abstract] The plant cell wall (PCW) is a pecto-cellulosic extracellular matrix that envelopes the plant cell. By integrating extra-and intra-cellular cues, PCW mediates a plethora of essential physiological functions. Notably, it permits controlled and oriented tissue growth by tuning its local mechano-chemical properties. To refine our knowledge of these essential properties of PCW, we need an appropriate tool for the accurate observation of the native (*in muro*) structure of the cell wall components. The label-free techniques, such as AFM, EM, FTIR, and Raman microscopy, are used; however, they either do not have the chemical or spatial resolution. Immunolabeling with electron microscopy allows observation of the cell wall nanostructure, however, it is mostly limited to single and, less frequently, multiple labeling. Immunohistochemistry (IHC) is a versatile tool to analyze the distribution and localization of multiple biomolecules in the tissue. The subcellular resolution of chemical changes in the cell wall component can be observed with standard diffraction-limited optical microscopy. Furthermore, novel chemical imaging tools such as multicolor 3D dSTORM (Three-dimensional, direct Stochastic Optical Reconstruction Microscopy) nanoscopy makes it possible to resolve the native structure of the cell wall polymers with nanometer precision and in three dimensions.

Here we present a protocol for preparing multi-target immunostaining of the PCW components taking as example *Arabidopsis thaliana*, Star fruit (*Averrhoa carambola*), and Maize thin tissue sections. This protocol is compatible with the standard confocal microscope, dSTORM nanoscope, and can also be implemented for other optical nanoscopy such as STED (Stimulated Emission Depletion Microscopy). The protocol can be adapted for any other subcellular compartments, plasma membrane, cytoplasmic, and intracellular organelles.

Keywords: Plant cell wall, Immunohistochemistry, Cell wall polysaccharides, Super-resolution microscopy, 3D dSTORM nanoscopy, Morphogenesis

[Background] PCW is an intricate material, which is durable but also undergoes constant structural changes in response to internal and external stimuli such as tissue expansion or pathogen attack. Nevertheless, how these seemingly contradictory features, mechanical strength and structural adaptation, cooperate, remains an unresolved question in plant cell biology. PCW contains cellulose,

hemicellulose, different variants of pectin and various proteins, the architecture of which is highly organized (Peaucelle, 2018). The pectin family is composed of several polymers. The most abundant, homogalacturonan, can be demethylated after cell wall insertion. This change in chemistry is a significant step in the process of cell elongation, differentiation, and directional growth (Peaucelle *et al.*, 2012). Several lines of evidence suggest that morphogenesis and cell differentiation are dependent on local changes in cell wall chemistry and cell wall polymer organization (Yang *et al.*, 2016; Anane *et al.*, 2017; Zhao *et al.*, 2019; Haas *et al.*, 2020). Therefore, detailed knowledge of PCW components architecture is essential for understanding plant growth. Historically, the cell wall structure has been studied using biochemical methods that involve disintegrating the tissue and destroying the native organization of its polymers (Höfte and Vioxeur, 2017). Other imaging modalities, such as electron microscopy (EM) (Anane *et al.*, 2017) and atomic force microscopy (AFM) (Zhang *et al.*, 2017) are used for *in situ* cell wall observation, but these techniques often lack chemical contrast, providing only correlative quantification. Multicolor immunohistochemistry (IHC) can reveal multiple targets within a tissue section, and their spatial organization resolved in three dimensions. Despite its widespread use in the cell biology field, multicolor IHC is not yet a standard tool for studies of the cell wall. A broad palette of antibodies and probes against cell wall targets, coupled with multicolor IHC, together with the high resolving power of dSTORM (< 40 nm), permits quantitative *in situ* chemical analysis of cell wall nanostructure. Other imaging techniques for cell wall analysis on tissue cuts exist, such as Raman (Wightman *et al.*, 2019) and FTIR (Mravec *et al.*, 2017; Cuello *et al.*, 2020). These techniques are based on the characteristic absorption/transmission of different chemical components and can measure some changes in cell wall composition at the cellular level but can lack sensitivity, and their ability to observe changes at the subcellular level is severely limited.

The dSTORM permits localization of biomolecules with the precision of 5-10 nm; however, the final dSTORM resolution, typically around 40 nm, is limited by the size of the antibody complex (~15-30 nm). The immunogold Electron Microscope (iEM) is also used in combination with ICH to study cellular structures at high resolutions. IEM is comparable to dSTORM resolution and is also limited by the antibody complex size. The size of the nanogold particle defines iEM contrast and resolution; > 1 nm gold nanoparticles are available; however, such a small particle limits the contrast, and larger particles are often used. IEM, contrasted with dSTORM, has several additional drawbacks: (1) the primary antibodies probed with protein-A (or G) gold complexes do not penetrate through the resin-embedded sample, and only recognize the surface epitopes, although the serial and ultra-thin cryo-sectioning technique can resolve this problem (50 nm, -120 °C [Slot, 1989]); (2) samples are mostly single-labeled; yet by using different nanoparticle size, two or three epitopes can be tagged, but it requires the technical experiences of ultra-thin cryo-sectioning technique (Slot and Geuze, 2007); (3) IEM has low labeling and detection efficiency (3-5 orders of magnitude less than dSTORM (Majda *et al.*, 2017; Haas *et al.*, 2020), which is also related to the fact, that only the surface epitopes are labeled. Multicolor 3D dSTORM nanoscopy can, therefore, provide unprecedented insights into the nanoarchitecture of the native-structure of the cell wall polysaccharides, beyond the applicability of the aforementioned techniques. DSTORM permits a quantitative three-dimensional (3D) nanoimaging of cells and tissues (Heilemann

et al., 2008; Huang *et al.*, 2008; Van De Linde *et al.*, 2011; Sydor *et al.*, 2015; Xu *et al.*, 2018). Applied mainly to cellular systems, it unveiled new structural organizations of proteins, e.g., synaptic nanodomains, trans-synaptic nanocolumns, DNA recombinase nanofilaments, nuclear envelope pore structure, cytoskeleton, mitochondria, adhesion complexes, and chromatin transcriptional landscape (Shroff *et al.*, 2008; Shim *et al.*, 2012; Löschberger *et al.*, 2012; Jakobs and Wurm, 2014; Prakash *et al.*, 2015; Boettiger *et al.*, 2016; Sellés *et al.*, 2017; Dlasková *et al.*, 2018; Haas *et al.*, 2018a and 2018b; Pan *et al.*, 2018; Xia *et al.*, 2019; Chen *et al.*, 2020; Wäldchen *et al.*, 2020). Yet, due to the limitation of single-cell model systems requiring tissue-level imaging, its utilization in plant science is almost absent (Liesche *et al.*, 2013; Komis *et al.*, 2015; Haas *et al.*, 2020). Here we present a detailed protocol for the sample preparation compatible with a standard confocal microscope and a dSTORM nanoscope on thin plant tissue sections.

Materials and Reagents

38. 8-well ibidi µ-slides for dSTORM sample preparation and imaging (Ibidi, catalog number: 80827)
39. Biopsy cassette 42 x 28 x 6 mm (Leica Biosystems, ID: IP-Biopsy-cassette-III)
40. Metal base mold (Leica Biosystems, ID: metal-base-molds)
41. Charged slides by poly-L-lysine treatment for confocal imaging sample preparation and imaging such as ProbeOn Plus (Fisher Scientific catalog number: 22-230-900 or Polysine™ Microscope Adhesion Slides (Thermo Scientific catalog number: J2800AMNZ))
42. Aluminum foil (you can get it from a local store)
43. Coverglass, rectangular, 24 x 60 mm, #1.5 thickness (Knittel Glass, catalog number: 425-2460)
44. Eppendorf Safe-lock Microcentrifuge Tubes, 1.5 ml or 2 ml volume (Eppendorf, catalog numbers: 022-36-320-4 [1.5 ml], 022-36-335-2 [2 ml])
45. Cardboard Slide Tray (Heathrow Scientific, catalog number: HD9902)
46. Paper towels (Divers/Dutcher, catalog number: 475040)
47. Empty pipette tips box for homemade humidified chamber for ibidi µ-slides, such as Adamas-Beta 1,000 µl volume tips
48. Nitrile gloves, such as Fisherbrand™ Comfort Nitrile Gloves (Fisher Scientific, catalog number: 15642367)
49. National Diagnostics™ Histoclear™ Tissue clearing agent (Fisher Scientific, HS-200-1GAL, CAS number: 5989-27-5)
50. Embedding medium paraffin (Leica Biosystems, ID: em-400-embedding-medium-paraffin)
51. Oxoid Skim Milk Powder (Thermo Fisher Scientific, catalog number: LP0031B)
52. CMB3a Crystalline cellulose-binding module, his-tagged and recombinant CBM protein (PlantProbes, catalog number: CMB3a) (Blake *et al.*, 2006; Hernandez-Gomez *et al.*, 2015), store at -20 °C
53. Mouse monoclonal antibody against low esterified homogalacturonan (degrees of methyl-esterification (DM) up to 40%) 2F4 (PlantProbes, catalog number: 2F4) (Liners, Thibault and

Van Cutsem, 1992), store at 4 °C

54. Rat IgM monoclonal antibody against high-esterified homogalacturonan LM20 (PlantProbes, catalog number: LM20) (Verhertbruggen *et al.*, 2009), store at 4 °C
55. Rat IgA monoclonal antibody against partially methyl-esterified epitopes of homogalacturonan (PlantProbes, catalog number: JIM7) (Knox *et al.*, 1990), store at 4 °C
56. Rat IgG2a monoclonal antibody against xyloglucan binding preferentially to the XLLG motif of xyloglucan LM24 (PlantProbes, catalog number: LM24) (Tanackovic *et al.*, 2016), store at 4 °C
57. Anti-His tag polyclonal antibody produced in chicken, Anti-6X-His tag (Abcam, catalog number: ab9107) store at -20 °C
58. Anti-His tag polyclonal antibody produced in rabbit (Merck, catalog number: SAB4301134), store at -20 °C
59. Rabbit PDM antibody against Mannan was a kind gift by Paul Dupree (Handford *et al.*, 2003; Yang *et al.*, 2016), store at 4 °C. For availability, please contact raymond.wightman@slcu.cam.ac.uk
60. Recombinant and His-tagged CBM4 of *Cellulomonas fimi* (ATCC 484) endoglucanase C (CBD4_{N1}) produced from *E. coli*. CBM4 was a kind gift by Harry Gilbert (Johnson *et al.*, 1996; Blake *et al.*, 2006), store at 4 °C. For availability, please contact raymond.wightman@slcu.cam.ac.uk
61. Goat anti-rat CF568 secondary antibody (Sigma, catalog number: SAB4600086), store at -20 °C
62. Goat anti-mouse CF568 conjugated F(ab')2 secondary antibody fragment (Sigma, catalog number: SAB4600400), store at -20 °C
63. Goat anti-mouse F(ab')2 secondary antibody fragment conjugated to Alexa Fluor 647 (Stratech Scientific, catalog number: 115-607-003-JIR), store at 4 °C
64. Donkey anti-mouse F(ab')2 secondary antibody fragment conjugated to Alexa Fluor 647 (Abcam, catalog number: ab181292), store at 4 °C
65. Donkey anti-Chicken F(ab')2 secondary antibody fragment conjugated to Alexa Fluor 647 (Jackson immunoresearch, catalog number: 703-606-155), store at 4 °C
66. Goat anti-mouse F(ab')2 secondary antibody fragment conjugated to ATTO488 (Hypermole, catalog number: 2402-0.5MG), store at -20 °C
67. Goat anti-mouse Alexa 488 conjugated secondary antibody (Sigma, catalog number: SAB4600387), store at 4 °C
68. Donkey anti-rat F(ab')2 secondary antibody fragment conjugated to Alexa Fluor 647 (Abcam, catalog number: ab150151), store at 4 °C
69. ProLong Gold Antifade Mountant (Thermo Fisher Scientific, catalog number: P36934), store at 4 °C
70. Ammonium chloride (NH₄Cl) (Sigma-Aldrich, catalog number: 254134), store at RT
71. Poly-L-lysinesolution 0.1% (w/v) in H₂O (Merck, catalog number: P8920), store at RT
72. Ethanol absolute anhydrous (CARLO ERBA reagents, catalog number: 4146082), store at RT
73. Acetic Acid ≥ 96% (AnalR NORMAPUR, catalog number: 20099.324), store at RT

74. Formaldehyde (Sigma-Aldrich catalog number: F1635), store at RT
75. *Arabidopsis* inflorescence meristem fixed at 1 cm length stage, and cotyledon of 3 days-old seedling, leaf rachis of star fruit (Supplemental Figure 1AG), immature maize leaf blade at 6th node (Supplemental Figure 1AF)
76. Nail polish (you can get it from a local drugstore)
77. Pectolyase for enzymatic extraction of pectins (Sigma, catalog number: P3026 or Magazyme, catalog number: E-PLYCJ)
78. Calcofluor White Stain for general cell wall staining (Sigma Aldrich, catalog number: 18909-100ML-F)
79. 2F4 Buffer (T/Ca/S buffer final concentration) (see Recipes), store at RT
80. FAA solution (see Recipes), store at RT
81. Formaldehyde diluted in 10x 2F4 buffer (see Recipes)
82. Citrate-Phosphate Buffer for Pectolyase incubation (pH 4.8) (see Recipes)

Equipment

13. Dispensable microtome knife (Microm Microtech, catalog number: F/MM35p)
14. Micro tweezer (Ideal-tek, catalog number: 5-SA)
15. Glass Rectangular 250 ml Coplin Staining Jar, with Lid (Wheaton, catalog number: 900620)
16. Fisherbrand™ Microscope Slide Box for homemade humidified chamber for Polysine microscope slides (Fisher Scientific, catalog number: 22363400)
17. Brush for the capture of serial cuts obtained by the microtome (Supplemental Figure 1K, any fine brush from local stationery store, such as Etude, P10531.00, #2)
18. HistoCore Arcadia Heated Paraffin Embedding Station (Leica, ID:14039357258)
19. Leica EG F Electric Heatable Forceps
20. Microtome (Leica, model: RM2265)
For more information, see [user's manual](#).
21. Fridge 4 °C
22. Incubation chamber, such as Selecta (set at 60 °C), airflow not necessary
23. Laboratory freezer (-20 °C), such as Kirsch FROSTER LABO 330 ULTIMATE
24. Electronic laboratory heating plate (BIO-OPTICA Milano SpA, catalog number: 40-300-300)
25. Laboratory chemical fume hood

For the imaging steps:

26. Any Fluorescence Microscope equipped with four laser lines for the detection in UV (405 nm), green (488 nm), red (561 nm) and far red (633 nm) (here we use Zeiss LSM 710, Zeiss Oberkochen Germany)

Brochure available [here](#).

Specification

- a. Stands: Inverted (Axio Observer Z1 with side port port)
- b. Z drive and XY stage (option): Motorized XY-scanning stage, with Mark & Find function (xyz) and Tile Scan (mosaic scan); the smallest increments 1 μm
- c. Objectives: x10, x25, x40, x63, x100
- d. Lasers: Argon laser (458, 488, 514 nm), HeNe laser (633 nm), diode laser 405 nm, and DPSS laser 561 nm
 - i. 405 nm for Calcofluor White
 - ii. 488 nm for Alexa 488 and ATTO 488
 - iii. 561 nm for CF568 and Alexa 568
 - iv. 633 nm for Alexa 647
- e. Scanning Module
 - Model: Scanning module with 32 spectral detection channels (QUASAR)
 - Scanners: Two independent, galvanometric scan mirrors with ultra-short line and frame fly back.
 - Scanning resolution: 4 x 1 to 6,144 x 6,144 pixels
 - Scanning speed: 8 frames/sec with 512 x 512 pixels.
 - Number of fluorescence-spectral detectors: 2
 - Bright field transmission detector: Installed
- f. Software
 - Standard Software: ZEN2010
 - Optional Softwar: Image J or Fiji
 - Computer specification: HP Z800 Workstation, 64-bit Windows 7 Ultimate 2009, 24 Gb RAM, Intel® Xeon® CPU, X5650, Two processors 2.67 GHz, 2.66 GHz
 - Wild-field microscope (Nikon N-STORM)

Software

6. Grafeo (Custom made software for dSTORM data analysis and visualization, <https://github.com/inatamara/Grafeo-dSTORM-analysis-> (Haas et al., 2018b)
7. Fiji (<https://imagej.net/Fiji/Downloads>)

Procedure

In this protocol, we use *Arabidopsis thaliana* cotyledons meristem, star fruit (*Averrhoa carambola*) rachis (central fiber in the compound leaf), and maize leaf samples.

A. Sample Preparation

1. Star fruit was grown in soil in the greenhouse for two years, and leaf rachis was harvested at the beggining of April 2019. Maize was grown in soil in the field sown in May and harvested at the beginning of July 2019 before its flowering. *Arabidopsis* was grown on MS (Murashige and

Skoog) solid nutrient agar medium without sucrose, in constant light at 21 °C, and seedlings were harvested at three days after germinations (3 DAG) (Peaucelle, Wightman and Höfte, 2015). *Arabidopsis* meristem was harvested from a plant grown on soil in the growing chamber and harvested when the inflorescence was 1 cm long; flowers with visible sepals were removed, keeping all the closest flower buds (as described in Yang *et al.*, 2016).

2. Leaf rachis of star fruit was cut with a dispensable microtome knife into 0.5 cm-length-explant (Supplemental Figures 1AG, 2A). The immature region of the Maize shoot tip was isolated from the plantlet (Supplemental Figure 1AF). Maize immature leaf-blade without midrib at 6th node was cut into 5 mm x 5 mm square (Supplemental Figure 2C).
3. A volume range to fix different organs of *Arabidopsis* is typically between 1:10 to 1:100 (tissue to fixative volume), here we placed 20 seedlings without the excision of cotyledon in 1.5 ml Eppendorf tube filled with a 1.0 ml of FAA solution. The volume ratio for maize leaf explants was 1:3, and the volume ratio of star fruit leaf rachis explants was 1:100. We placed 5 explants of maize leaves and 10 explants of star fruit leaf rachis in 2.0 ml Eppendorf tube filled with 1.5 ml of FAA solution, respectively. The significant protocol steps are shown in the [Supplemental Figure 1](#), and the preparation of tissue explants, and the microtome cutting position in [Supplemental Figure 2](#).

B. Fixation and sample embedding

1. Wear nitrile gloves.
2. Fix plant organs in the FAA solution in the 1.5/2 ml Eppendorf tube for 1 h at room temperature or overnight at 4 °C. Store fixed samples at 4 °C for up to 1 month in 70% EtOH (see Notes). No vacuum treatment is necessary.
3. Dehydrate the samples by incubating at room temperature in successive ethanol dilutions for at least 30 min each: 70%, 95%, and twice 100% ethanol. Use approximately 1.5 ml volume in the Eppendorf tube.
4. Replace ethanol with 1.5 ml of 50% HistoClear in ethanol in Eppendorf for 1 h at room temperature, followed by 1.5 ml of 100% HistoClear for 1 h each at room temperature (this time depends on the thickness of the sample. Reduce the incubation time for very thin tissue cuts, such as roots or Hypocotyl to 30 min).
5. Transfer the sample to a biopsy cassette (IP-Biopsy-Cassette-III, Leica Biosystems). Start to melt the paraffin in an incubation chamber preheated to 60-70 °C, 12 h prior to Procedure B. Completely melted paraffin and new HistoClear should be preheated to 60-70 °C prior to Step B6 to make 50% paraffin in HistoClear. During a typical experiment, we use approximately 600 ml of paraffin and 100 ml HistoClear in Step B6.

Preheat the 250 ml glass jar containing 200 ml of the final of 50% HistoClear and 50% paraffin before Step B6.

Note: We recommend using the EM-400 Embedding Medium Paraffin, which has a low melting point (56-57 °C). This helps the precise positioning of the sample and reduces tissue distortion.

6. Replace the HistoClear with the paraffin in the incubation chamber by immersing biopsy cassettes in a 250 ml Coplin glass staining jar (Supplemental Figure 1B). Start with a mixture of 50% HistoClear and 50% paraffin in 200 ml for 3 h at 60-70 °C, followed by twice 200 ml of 100 % paraffin for 3 h, and finally 200 ml of 100% paraffin overnight at 60-70 °C.
7. Turn on the HistoCore Arcadia H Heated Paraffin Embedding Station (Supplemental Figure 1C) and set the HistoCore Arcadia H in operation mode before Step B8 (Set the temperatures of paraffin tank, dispenser, working surface at ~70 °C and a cold spot at 4 °C. For more information, See the HistoCore Arcadia H [user manual](#)).
8. Take out biopsy cassettes and hot 100% paraffin in 250 ml Coplin glass staining jar from the incubator. Let biopsy cassettes floating in melted paraffin of heated instrument tray on a hot preparation surface set at ~70 °C of HistoCore Arcadia H before the solidifying the paraffin at room temperature (Supplemental Figure 1D).
9. Transfer of the samples from the biopsy cassette to the metal base mold using electric heatable forceps (Supplemental Figure 1E). Samples should be positioned perpendicular to the cutting plane before solidifying the paraffin at room temperature (Supplemental Figures 1F to 1H and 2D).
10. Once positioned, rapid solidification of the paraffin could be achieved using a cold spot (small round metallic plate shown in Supplemental Figure 1G), usually at 4 °C.
Note: Rapid cooling of the paraffin will limit the formation of paraffin crystals, and make the preparation transparent. In contrast, slow cooling will lead to crystal formation and the widening of the preparation. The crystallized paraffin is slightly more rigid and limits greatly the ability to localize the sample during microtome cutting.
11. After Step B10, store the samples at 4 °C overnight. Before cutting samples with the microtome, keep the room temperature below 22 °C to prevent the paraffin softening.
12. Turn on the microtome. We recommend using the microtome in the manual mode. The use of the motorized mode is possible, but we did not test it. The settings for the cut thickness are: 3-5 µm (for more information: See the [user's manual of Leica](#), model: RM2265).
13. Take out the paraffin block of a tissue specimen from the mold manually, trim the paraffin block, and fix it in the specimen clamps and holder in the microtome (Supplemental Figure 1H). This step will assure a clean and homogenous cut with the formation of a straight ribbon, as shown in the Supplemental Figure 1K.
Trim the paraffin block to get the cutting surface of the specimen facing the knife with a microtome (Supplemental Figure 1I), if you need it.
14. Capture the sections by using a brush (Supplemental Figure 1K) and put them on a poly-L-lysine treated microscope slide (Supplemental Figure 1L) or 8-well ibidi µ-slides (Supplemental Figure 1M) without inverting sections, i.e., position tissue section side that was facing the knife (the "shiny" side) on the charged slide or ibidi well bottom glass.
15. Add a droplet of water between the slide and the serial tissue sections on the slide or a single ibidi well (Supplemental Figures 1N and O, see **Note below**).

Note: If you use the ibidi multi-well slides for dSTORM sample preparation, add 0.1% Poly-L-Lysine solution instead of water at this stage.

16. Keep the slides at 42 °C for 3 min.
17. Remove the water carefully without touching the cuts. Any drop of remaining water will form a bubble and will lead to the loss of this part of the sample. At this stage, manually spin-dry the slide by swinging it at arm's length 1 or 2 times (Supplemental Figure 1P).

Note: Wearing a face mask and gloves is necessary only during the pandemic.

18. Leave to dry at least overnight at 37 °C on a heating plate (Supplemental Figure 1Q). The slide can be conserved at room temperature for at least one month. Use the microscope slide boxes for the storage to avoid the dust.
19. Deparaffining (Supplemental Figure 1R): Place up to 8 micro slides vertically (or 16 slides back to back) into a 250 ml Coplin glass staining jar and immerse the slide in the three successive treatments with 200 ml of HistoClear each for 30 min, at RT in normal light condition, shaking is not necessary. Then wash the HistoClear-treated microslides with a 200 ml of 100% ethanol using a 250 ml glass staining jar for 20 min.

Note: The jar can be re-used at the next to the following step. Do not wash jar with water; just decant the solution from the jar.

20. Rehydrate the samples with successive treatments using the same two glass jar used in previous steps, 15 min each, in 100% ethanol, 70% ethanol, 50% ethanol, 25% ethanol, 10% ethanol in 2F4 buffer, and finally 100% 2F4 buffer, at RT in normal light condition, shaking is not necessary. Use approximately 200 ml volume for each step.

Note: The jar can be re-used at the next to the following step. Do not wash the jar with water; just decant the solution from the jar.

21. Proceed to Step C1 (Immunolabeling) as soon as possible to avoid the drying of sections.

Notes:

- a. *In the Step B12, the sample should be prepared in advance and kept at 4 °C overnight. However, samples can also be cut (Steps B12-B18) 1 h after Step B10. In such case, store the samples at -20 °C for 1 h prior to cutting and cut in less than 15 min after taking the sample from -20 °C.*
- b. *Use Eppendorf tubes to store tissues after fixation at 70% ethanol and dehydration in 100% ethanol.*

- C. Sample storage breakpoints: After the replacement to 100% HistoClear completely, the sample can be stored for a few days at RT. However, HistoClear is volatile and dissolves the paraffin; thus, it is not recommended for more than a few weeks. After fixation sample could be stored for months at 4 °C. After the embedding sample could be stored for years at a temperature below 25 °C. After cutting with microtome, the sample on microslides could be stored for several weeks at RT.

D. Immunolabeling

We present an example of multicolor immunostaining with 2F4 antibody against low methylesterified homogalacturonan binding a dimeric association of homogalacturonans through calcium ions, LM20 for high methylesterified homogalacturonan, JIM7 for partially methylesterified homogalacturonan, CBM3 and CBM4 to recognize mostly crystalline and amorphous cellulose respectively, PDM recognizing mannans, and LM24 recognizing xyloglucans. For all the steps, use 2F4 buffer instead of the PBS, even when not using the 2F4 antibody. It has proven to work correctly with different LM and JIM antibodies, for both CMB3 and CMB4 and microtubule antibodies (for the list of available LM, JIM and others antibodies and plant probes, please look here: www.plantprobes.net) using tissue cut samples of *Arabidopsis*, rice, star fruit, and maize (Yang et al., 2016). Importantly, if at any stage you wash out this buffer with water or PBS you will lose the 2F4 antibody staining.

Notes:

- a. *Prior to immunostaining, optionally, quench the free aldehyde groups using 50 mM NH₄Cl in 2F4 buffer for 15 min. Wash 3 times with 2F4 buffer, each time 3-5 min. This step is recommended since any residual aldehyde group from FAA solution used at Sample Fixation Step B2 will react with the amino group of the antibodies leading to unspecific antibody binding.*
- b. *We recommend applying all the primary antibodies successively as described in Steps C1-C5, to prevent competition and steric hindrance effects, especially for the closely located epitopes, such as cell wall targets presented here (cellulose, xyloglucan, pectin and mannan, Figure 1). However, performing simultaneous primary antibody incubation works well in most cases.*
- c. *To avoid the unspecific antibody binding, we use 5% milk in the 2F4 buffer as a blocking buffer. However, the milk can be contaminated over time; therefore, complete the immunostaining protocol Steps C1-C5 in less than 72 h, and where necessary, perform the overnight antibody incubation at 4 °C. Perform short antibody incubation and washing steps at room temperature (RT).*
- d. *Time schedule for staining. For the thin tissue sections (< 5 µm) 2 h at RT primary antibody incubation time is sufficient. For the thicker cuts and for certain antibodies, this time is extended to a minimum 3 h.*
- e. *Microwave Treatment. Depending on the manufacturer, selected antibodies may need microwave heating for activation or reaction acceleration (1 to 2 min at 400 watts). This step should be fast to prevent sample boiling—none of the antibodies presented in this protocol display improved efficiency of labeling after microwave heating.*
- f. *For enzyme treatments in the case of *Aspergillus* pectolyase from Sigma, treat sections with 200 µl of pectolyase in the incubation buffer (see Recipes) at the final dilution 0.1% at room temperature for 10 min prior to Step C1 (CBM incubation) or Step C2 (primary antibody incubation). After the enzyme treatments, wash sections with 2F4 buffer 3 times each time 3-5 min. When you use another pectate lyase such as [Megazyme E-PLYCJ](#), check their publication list for the reaction condition.*
- g. *How to make the hand made humid chamber (Supplemental Figure 1U). Place 2-3 layers of*

paper towel on the bottom of an empty micropipette box (10 cm x 14 cm x 9.5 cm) or an empty box for the microscopy slide tray (3 cm x 8.5 cm x 21 cm) and add distilled water (~20 ml), so that paper towels are humid. Cover the box. Use aluminum foil to wrap the humid chamber. If you use micro slides for confocal imaging sample preparation, make sure that the sample on the microslide is not in contact with the wet paper towels. For this, we recommend a microscopy slide tray.

At each step, we use 200 μ l of the antibody-containing solution per coverslip (Supplemental Figure 1S), while we use 50 or 70-100 μ l of the antibody-containing solution per a single well of the 8-well ibidi slide (Supplemental Figure 1T, see also Step C8). For washing, we use approximately 500 μ l per coverslip or 200 μ l per single well of 2F4 buffer with 5% milk. Position the sections in the center of a droplet. On the droplet, the reagents (antibodies) are concentrated (a process known as coffee stain). Here we present the following optimal sequential antibody staining order. Optionally, prior to the antibody or reagent incubation, Steps C1 or C2, incubate your sample in a blocking buffer solution for 30 min. Then go directly to Step C1.

1. The cellulose-binding molecule (CBM3 or CBM4) incubation: Dilute 2/100 volume per volume (v/v) CBM3/4 reagent in blocking buffer and add to samples. Incubate for 2 h at RT or overnight at 4 °C in the humid chamber(Supplemental Figure 1U). After removal of CBM3 solution, wash 3 times for 5 min using the blocking buffer. If you perform overnight incubation at 4 °C, the next day, take the humid chamber for samples out of the fridge at least 30 min before the washing steps.
2. The first primary antibody incubation: Dilute 20/100 v/v 2F4 antibody in blocking buffer and add to samples. Incubate for 2 h at RT or overnight at 4 °C in the humid chamber. After removal of 2F4 solution, wash 3 times for 5 min using the blocking buffer. If you perform overnight incubation at 4 °C, the next day, take the humid chamber for samples out of the fridge at least 30 min before the washing steps.
3. The second primary antibody incubation: Dilute 10/100 v/v LM20 antibody in blocking buffer and add to samples. Incubate for 2 h at RT or overnight at 4 °C in the humid chamber. After removal of LM20 solution, wash 3 times for 5 min using the blocking buffer. If you perform overnight incubation at 4 °C, the next day, take the samples out of the fridge at least 30 min before the washing steps.
4. The third primary antibody incubation: Dilute 1/100 v/v rabbit or chicken anti-his tag antibody in blocking buffer and add to samples. Incubate for 2 h at RT or overnight at 4 °C in the humid chamber. After removal of the antibody solution, wash 3 times for 5 min using the blocking buffer. If you perform overnight incubation at 4 °C, the next day, take the humid chamber for samples out of the fridge at least 30 min before the washing steps.
5. The secondary antibody incubation: Dilute all of three secondary antibodies 1/100 v/v in the same blocking buffer as a secondary antibody-mixture. Incubate for 2 h at RT or overnight at 4 °C in the humid chamber. Incubate the secondary antibodies in the dark to avoid the photo-

bleaching. For this, wrap the humidified chamber in aluminum foil. After removal of the secondary antibody-mixture solution, wash 3 times for 5 min using the blocking buffer. If you perform overnight incubation at 4 °C, the next day, take the humid chamber for samples out of the fridge at least 30 min before the washing steps. The secondary antibody incubation could also be done sequentially (see **Troubleshooting** subsection), although in the case of Figures 1 to 4, all the secondary antibodies were added simultaneously.

Notes:

- a. **Steps C1-C5:** Perform all the antibody incubation steps in the humidified chamber.
- b. This protocol can be extended for more than three targets using the antibodies from another species, such as rabbit, guinea pig, horse, and human. However, imaging more than four colors on the confocal microscope requires additional laser lines (> 700 nm) or spectral unmixing procedure.
- c. Make negative control images without 1st, 2nd, and 3rd primary antibodies like Figure 1A (Only Step C5 with secondary antibody mixture, at least in the case of your first trial).
- d. For dSTORM imaging, from Step B14, we recommend using the multi-well Ibidi Glass Bottom µ-slides, such as 8-well slides (Ibidi), which can be easily inserted and clipped on many microscope stages. The multi-well slides are convenient for the preparation of several imaging conditions, reduce the quantity of reagent used, and improves the homogeneity of the immunostaining for the semi-quantitative analysis. For 8-well Ibidi slides, we recommend using 70-100 µl of reagent per well at Steps C1-C5 of immuno-labeling.

6. Post-Immuno fixation.

This step is important for the dSTORM nanoscopy samples and is not necessary for samples prepared for confocal imaging using mounting media. Since dSTORM samples, contrary to confocal microscopy samples, are not mounted in the mounting media, it reduces the thermal motion of the antibody complex and slows down the antibody dissociation from the epitope.

- a. Incubate the sample for 10 min in 3.7% formaldehyde (see Recipe 4) diluted in an appropriate amount of 10x 2F4 buffer and distilled water at RT. For this, place 200 µl drop of 3.7% formaldehyde solution on the sample located inside the ibidi multi-well slide. Perform this step under the fume hood and wear nitrile gloves.
- b. Wash 3 times for 3 min with the 300 µl of 2F4 buffer. Perform this step under the fume hood.
- c. Quench the formaldehyde with 70 µl drop of 50 mM ammonium chloride for 15 min. This step reduces the risk of contact with formaldehyde to your skin while handling the sample during the imaging. Dilute the ammonium chloride in 2F4 buffer (see Recipes). After incubation, wash briefly 3 times with the 300 µl drop of 2F4 buffer. You can also use other aldehyde quenching reagents containing amine groups, such as 50 mM of glycine in 2F4 buffer.

7. Sample mounting and storage. Only for confocal imaging.

Note: Do not perform this step for dSTORM sample preparation.

To extend the lifespan of the sample, mount the slide with a coverslip in mounting media containing antifade reagent. We suggest ProLong Gold Antifade Mountant (Thermo Fisher Scientific) (Supplemental Figure 1V). To prevent the formation of bubbles, place a droplet of mounting media on the coverslip, and gently lower it on the slide using a micro tweezer (Supplemental Figure 1W-AA). Seal the coverslip with nail polish to prevent drying (Supplemental Figure 1AB) and put sealed micro slides into Cardboard Slide Tray (Supplemental Figure1AC) and store in the dark at 4°C.

8. Sample storage. Only for dSTORM imaging.

After completion of Steps C6, add 500 µl of 2F4 buffer to each of 8 wells, seal with parafilm, wrap in the aluminum foil, and keep in a humid chamber at 4 °C as presented in Supplemental Figures 1AD and 1AE.

Note: You can perform this for confocal imaging, but then you cannot mount your sample in the antifade mountant; therefore, it is not recommended.

Choosing the fluorescence dyes.

For confocal imaging, we recommend using bright and photostable dyes, such as Alexa Fluor, ATTO, and CF series. For the dSTORM imaging, only certain dyes work. We recommend ATTO488, CF568, and Alexa647 dyes. For the dSTORM nanoscopy imaging, we recommend using F(ab')2 secondary antibody fragments or nanobodies, or, when possible, to conjugate the primary antibodies directly with the fluorophore of interest. For densely packed epitopes, we recommend using either single labeled secondary antibodies.

Troubleshooting

High background: Reduce secondary antibody concentration. The good practice is to perform the titration of both primary and secondary antibodies. If, for instance, the recommended antibody dilution is 1:200, a good starting point for the titration test is 1:50, 1:100, 1:200, 1:400, and 1:800 dilution. Before immunostaining, quench free aldehyde groups using 50 mM NH₄Cl in the 2F4 buffer for 15 min. Use serum in blocking buffer in which the secondary antibody has been raised, e.g., goat serum for antibodies produced in goat. Incubate the antibodies at 4 °C overnight prior to Steps C1 or C2.

A high degree of colocalization when it is not expected: Use sequential staining for all the secondary antibodies. If possible, use the same host species for all the secondary antibodies (For example, a set of anti-rat antibody, anti-rabbit antibody and anti-mouse antibody from goat and so on). To avoid off-target binding, use highly cross-adsorbed secondary antibodies. We recommend preparing a test sample for which only Step C5 is performed, which is the application of the secondary antibodies without primary antibodies.

Low signal: Increase the primary antibody concentration and/or incubation time. Try to use a fresh batch of the primary and secondary antibodies. Change the secondary antibody (conjugated-dye, host species, or producer for the same type of antibody). Increase the secondary antibody concentration (perform the titration-test at the 2, 4, and 6 fold increased antibody dilution) and/or

incubation time (perform tests at 2 h, 4 h, 6 h). Check the pH of the 2F4 buffer. If pH is not 8.0, adjust it.

Confocal imaging tips

1) Counterstaining

For the semi-quantitative analysis of the cell wall component with a confocal microscope, compare the samples from the same experimental preparations. If possible, consider the observation of counterstained sample with the third or fourth channel of the laser scanning confocal microscope, if the counterstaining intensity is expected not to change in different sample conditions. For the cell wall staining, use, for instance, calcofluor white stain. Add 200 µl of calcofluor white stain after the Step C5 at 5 mM concentration in 2F4 buffer and incubate for 5 min at room temperature. Wash briefly twice with 500 µl of 2F4 buffer before applying the antifade mounting media to the sample (Step C7). The counterstaining is useful to normalize the intensity data to size, e.g., area or the cell wall thickness. Prepare all the conditions you want to compare for each experimental preparation.

2) Operation of microscope

Switch on the laser 1h before imaging. During the imaging, make sure not to saturate the signal at the detectors for downstream quantification. Keep the laser power, and detector gains the same across the experiments and keep all other settings constant between comparable experiments. Additionally, in order to avoid the photobleaching during the optimization of the image quality before the final take, consider using a tissue sample or region with less scientific interest, but a similar signal level present on your slide, 1) Find the target point and focus the section in bright field. Differential interference contrast image (DIC) is more convenient to focus the image if DIC is installed to your microscope. 2) Optimize the image quality at the position near the final target point. 3) Stop scanning frequently during the optimization of the image quality. 4) As the initial settings for confocal laser scanning mode, 1 to 2% of laser power is a good starting point to focus on a sample. 5) Faster or maximal scan speed (Arbitrary level of scan speed: > 9 in the case of Zeiss LSM 710) is recommended to focus on the initial conditioning (Live button of ZEN software is convenient in Zeiss LSM 710).

Note: There are fifteen and arbitrary levels of scan speed in LSM710 and the absolute speed at maximum level “15” is 8 frame/s, according to Zeiss local office. When performing quantitative imaging, due to photobleaching, never take the final image of the same area twice. Therefore, move the X-Y-Z stage for the micro slide in a certain direction (from right to left and from front to back) to avoid the observation of the same point. For reliable quantification, 2-3 independent experiments (independent technical replicates) need to be carried out and compared. Perform a pilot experiment to determine the common parameters, such as gain and pixel dwell time, and to abolish/minimize any photobleaching. Sequentially image all the channels, always starting with the longest wavelength and ending with the shortest wavelength.

Data analysis

Figure 1 shows dual- and triple-color IHC of different cell wall epitopes, representing members of three different families of the cell wall polysaccharides: pectin (2F4, LM20, and JIM7), cellulose (CBM4, CBM3) and hemicellulose (PDM and LM24) in the transverse section of star fruit leaf rachis (Supplemental Figure 2, and Table 1).

Comparing Figure 1 panels A and C, the cellulose staining (CBM3 and CBM4, see Table 1) is weak in areas presenting high HG counts and high in tissues with low HG counts. This could suggest that cellulose and pectins occupy exclusive wall compartments. However, the enzymatic extraction of pectin revealed much higher cellulose detection levels with CBM4 (compare Figures 1C and 1E). This indicates that, in the intact cell walls, the majority of cellulose epitopes are masked by pectins. Without pectin extraction, the remaining cellulose staining may correspond to xyloglucans, which are partially detected by CBM probes (see PlantProbes CMB3 reagent description, Hernandez-Gomez *et al.*, 2015). Indeed, Figure 1D shows that CBM3 and LM24 antibody against xyloglucans present a low degree of overlap in the subset of walls.

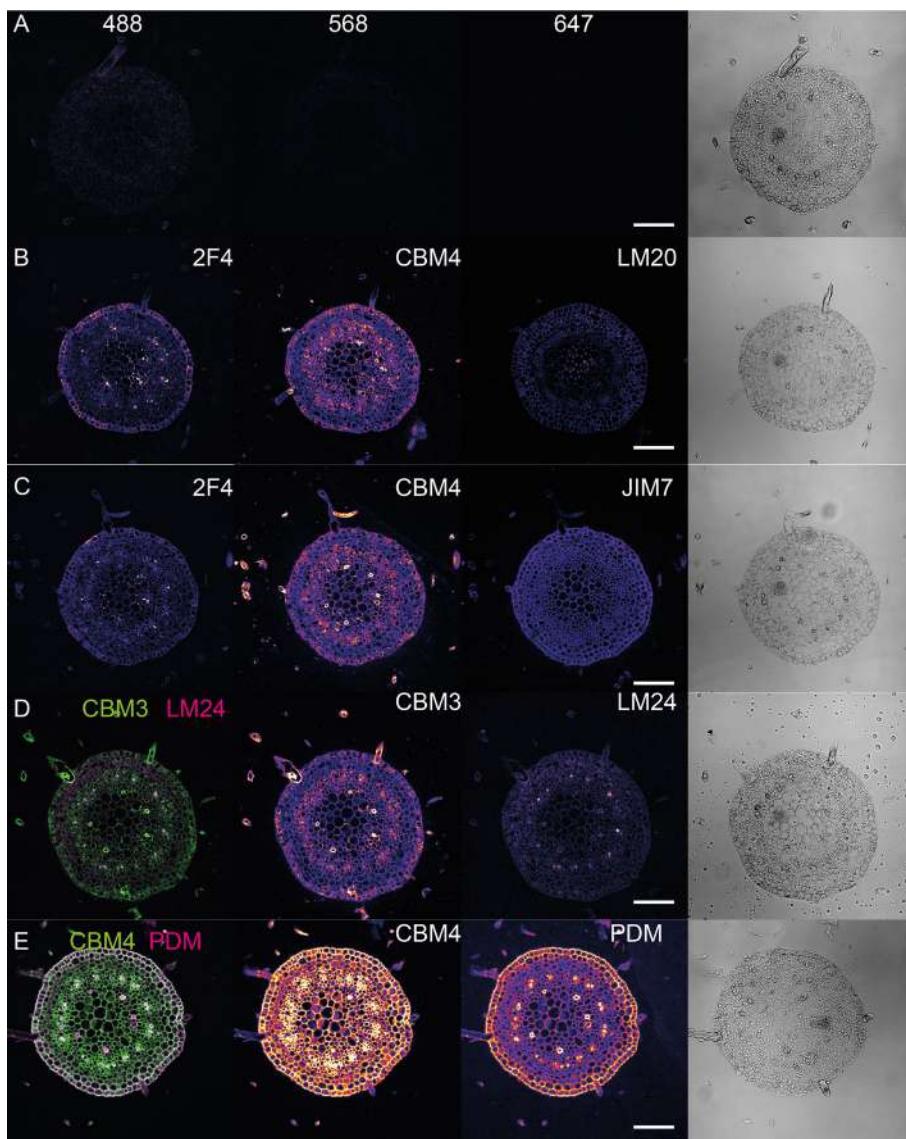


Figure 1. Multicolor confocal imaging of different cell wall epitopes in the star fruit (*Averrhoa carambola*) leaf rachis. A. Control images with only the secondary antibody staining. B. Triple staining of 2F4, CBM4 and LM20, and (C) 2F4, CBM4, and JIM7 epitopes. D. Double staining of CBM3 and LM24. E. Double staining of CBM4 and PDM after enzymatic pectin extraction. All the images were acquired with the same microscope settings (see Table 2 for suggested settings). The last column represents transmission images of the cuts. Images were visualized in Fiji. Scale bars, 100 μ m.

Table 1. The primary antibody, CBM reagents, and the secondary antibody dilutions used in Figures 1-4

Figure Number	The primary antibody or CBM reagent	Secondary Antibody
Figure 1A (Control)		Anti-Chicken CF568 1/100
		Anti-Mouse Alexa 488 1/100
		Anti-Rat Alexa 647 1/100
Figure 1B	CBM4-His 5/100	
	Anti-His/Chicken 2/100	Anti-Chicken CF568 1/100
	2F4 20/100	Anti-Mouse Alexa 488 1/100
	LM20 10/100	Anti-Rat Alexa 647 1/100
Figure 1C	CBM4-His 5/100	
	Anti-His/Chicken 2/100	Anti-Chicken CF568 1/100
	2F4 20/100	Anti-Mouse Alexa 488 1/100
	JIM7 10/100	Anti-Rat Alexa 647 1/100
Figure 1D	CBM3-His 5/100	
	Anti-His/Chicken 2/100	Anti-Chicken CF568 1/100
	LM24 10/100	Anti-Rat Alexa 647 1/100
Figure 1E	CBM4-His 5/100	
	Anti-His/Chicken 2/100	Anti-Chicken CF568
	PDM 5/100	Anti-Rabbit Alexa 647 1/100
Figure 2A	2F4 20/100	Anti-Mouse Alexa 647 1/100
	JIM7 10/100	Anti-Rat CF568 1/100
Figure 2B	2F4 20/100	Anti-Mouse Alexa 647 1/100
	LM20 10/100	Anti-Rat CF568 1/100
Figure 3A	2F4 20/100	Anti-Mouse Alexa 647 1/100
	JIM7 10/100	Anti-Rat CF568 1/100
Figure 3B	2F4 20/100	Anti-Mouse Alexa 647 1/100
	JIM7 10/100	Anti-Rat CF568 1/100
Figure 3C	CBM3-His 5/100	
	2F4 20/100	Anti-Mouse Alexa 647 1/100
	Anti-His/Chicken 2/100	Anti-Rat ATTO 488 1/100
Figure 4A	CBM4-His 5/100	
	Anti-His/Chicken 2/100	Anti-Chicken Alexa 647 1/100
	PDM 5/100	Anti-Rabbit CF568 1/100
Figure 4B	CBM3-His 5/100	
	Anti-His/Chicken 2/100	Anti-Chicken Alexa 647 1/100
	PDM 5/100	Anti-Rabbit CF568 1/100

Table 2. The suggested emission filter bandwidth for the triple immunostaining and a counterstaining with calcofluor white using confocal laser scanning microscope optimized to minimize spectral crosstalk. For details on the filter sets for dSTORM please refer to Haas *et al.* (2020)

Dye name	Emission Filter bandwidth	Excitation wavelength
Alexa 488/ATTO 488	510-550 nm	488 nm
CF568/Alexa 568	580-640 nm	568 nm
Alexa 647	660-760 nm	642 nm
Calcofluor White	420-480 nm	405 nm

Figure 2 shows the 3D dSTORM nanoscopy imaging of the different pectin species in the L2 layer (layer below the epidermis) of the *Arabidopsis* cotyledon with the longitudinal section described in Supplemental Figure 2E. In Figures 2A and 2B, the typical cell edge staining of low-methylated (2F4), high-methylated (LM20), and partially methylated (JIM7) pectin is observed (see Table 1). The 3D dSTORM images revealed that the staining is weak inside the cell wall (middle lamella) and is mostly limited to the cell wall edges facing the cytoplasm—this highlights one of the biggest limitations of IHC in general: the epitope accessibility and antibody penetrability. Yet, in light of available techniques that possess similar spatial and chemical resolution (e.g., iEM), 3D dSTORM provides overall higher labeling and detection density, and the number of epitopes detected in the middle lamella remains higher for 3D dSTORM than iEM (Cosgrove and Anderson, 2020). One possible way to bypass this limitation is to remove one polymer (for example, structural protein degraded with the recombinant Protease K) from the cell wall to increase antibody penetration. However, this may cause an unspecific wall perturbation. The development of small nanobodies against the cell wall targets will help in the future to address these limitations. The advance in correlative super-resolution light and electron microscopy may help enhance the performance and annihilate the limitations of 3D dSTORM and EM.

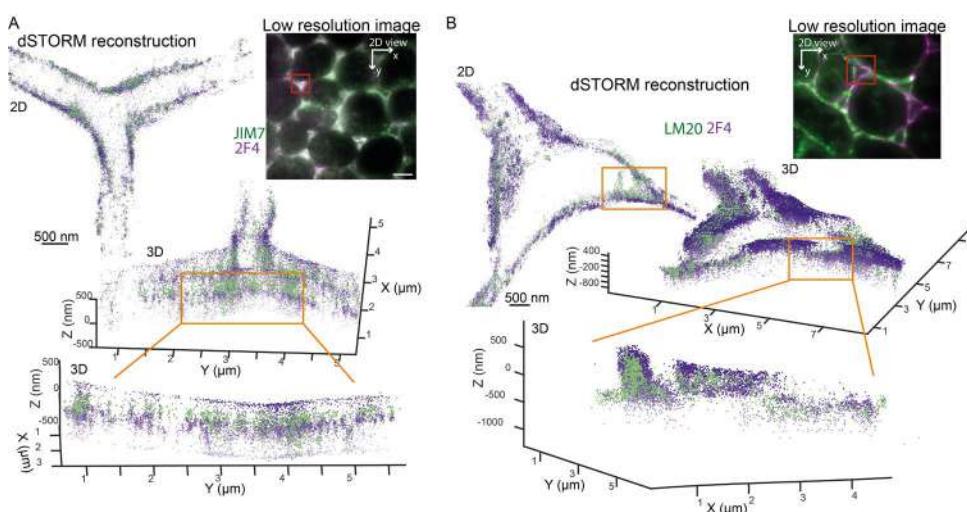


Figure 2. Two-color 3D dSTORM imaging of Homogalacturonan at tri-cellular junctions of L2 (subepidermal cell) layer in *Arabidopsis* cotyledon. Two-color scatter plots showing the 3D coordinates of localized (A) JIM7 (green) and 2F4 (violet), and (B) LM20 (green) and 2F4 (violet) epitopes. Image insets show a low-resolution oblique illumination image with red square outlining regions shown in the scatter plots. 2D—two dimensional, top view (XY), and 3D—three dimensional, inclined view, where the Z-axis is oriented perpendicular to the conventional image plane. The orange lines show the cell wall region enlarged in the panel below. Image scale bar, 4 μm . Data were visualized using GrafeoV.2.

Figure 3 shows the 3D dSTORM nanoscopy detection of the different pectin species in the epidermal layer of the *Arabidopsis* cotyledon with a longitudinal section described in Supplemental Figure 2G. In both Figures 3A and 3B, 2F4 and LM20 epitopes form a filamentous pattern of staining in the anticlinal, but not periclinal walls, described previously as the HG nanofilament (Haas et al., 2020). This nanofilament quaternary structure is challenging the canonical view on the pectin *in muro* architecture as an amorphous gel-like matrix. These unexpected results raise two concerns: (1) that the filamentous pattern represents pectins as a spacer between cellulose microfibrils, or (2) that pectin antibody cannot penetrate between the grooves formed by the cellulose microfibrils (Cosgrove and Anderson, 2020). To address these concerns, we labeled partially methylated HG epitope (JIM7) in the anticlinal walls of the cotyledons, Figure 3A. JIM7 presents a much broader and uniform distribution pattern compared to LM20 and 2F4 HG epitopes. First, the diffuse distribution of JIM7 epitope compared with LM20/2F4 filamentous distribution suggests that the pectin methylation pattern somehow correlates with its localization. Secondly, it implies that HG nanofilaments of LM20 and 2F4 are likely, not due to epitope inaccessibility in the grooves formed by cellulose microfibrils, as JIM7 epitope can be localized there, Figure 3A. Moreover, double staining for cellulose (CBM3) and HG (2F4) in Maize epidermal cells of an immature leaf at 6th node (longitudinal section in Supplemental Figures 2C and F), without prior extraction of pectins, shows that empty spaces between 2F4 nanofilaments are not filled with cellulose and that the overall level of the cellulose staining without pectin extraction is very low, Figure 3C.

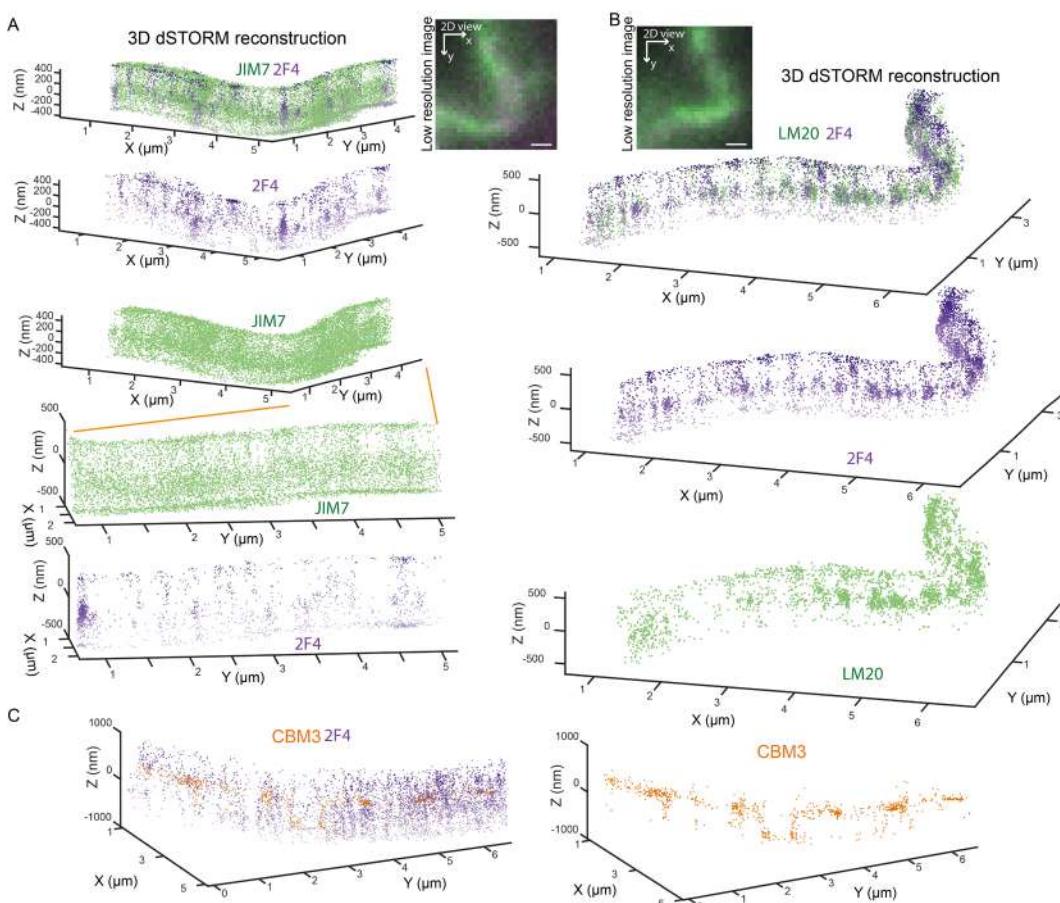


Figure 3. Two-color 3D dSTORM imaging of Homogalacturonan at the lobes of *Arabidopsis* cotyledons pavement cells and maize leaf pavement cells. Two-color scatter plots showing the 3D coordinates of localized (A) JIM7 (green) and 2F4 (violet), and (B) LM20 (green) and 2F4 (violet) epitopes in the *Arabidopsis* cotyledons. Image insets show a low-resolution fluorescence image of the regions shown in the 3D dSTORM scatter plots. The orange lines show the cell wall region enlarged in the panels below. (C) Two-color scatter plots showing the 3D coordinates of localized 2F4 (violet), and CBM3 (orange) epitopes in the Maize leaf. Scale bar, 1 μ m. Data were visualized using GrafeoV.2.

Figure 4 shows two-color 3D dSTORM imaging of *Arabidopsis* primordia and meristem directed towards cellulose (CBM3 or CBM4) and (hetero)mannan (PDM) and with prior pectin extraction with a longitudinal section of 1 cm of the inflorescence (Supplemental Figures 2B and 2D, Table 1). Comparing panels A and B show that CBM3, detecting predominantly crystalline cellulose, forms discreet filaments distribution as contrasted with CBM4, tagging amorphous cellulose. The staining for mannans displays different abundance in different cell walls: from extended wall sections mostly exclusive from the cellulose (Figure 4A) to punctate staining decorating extremities of the cellulose nanofibrils (Figure 4B).

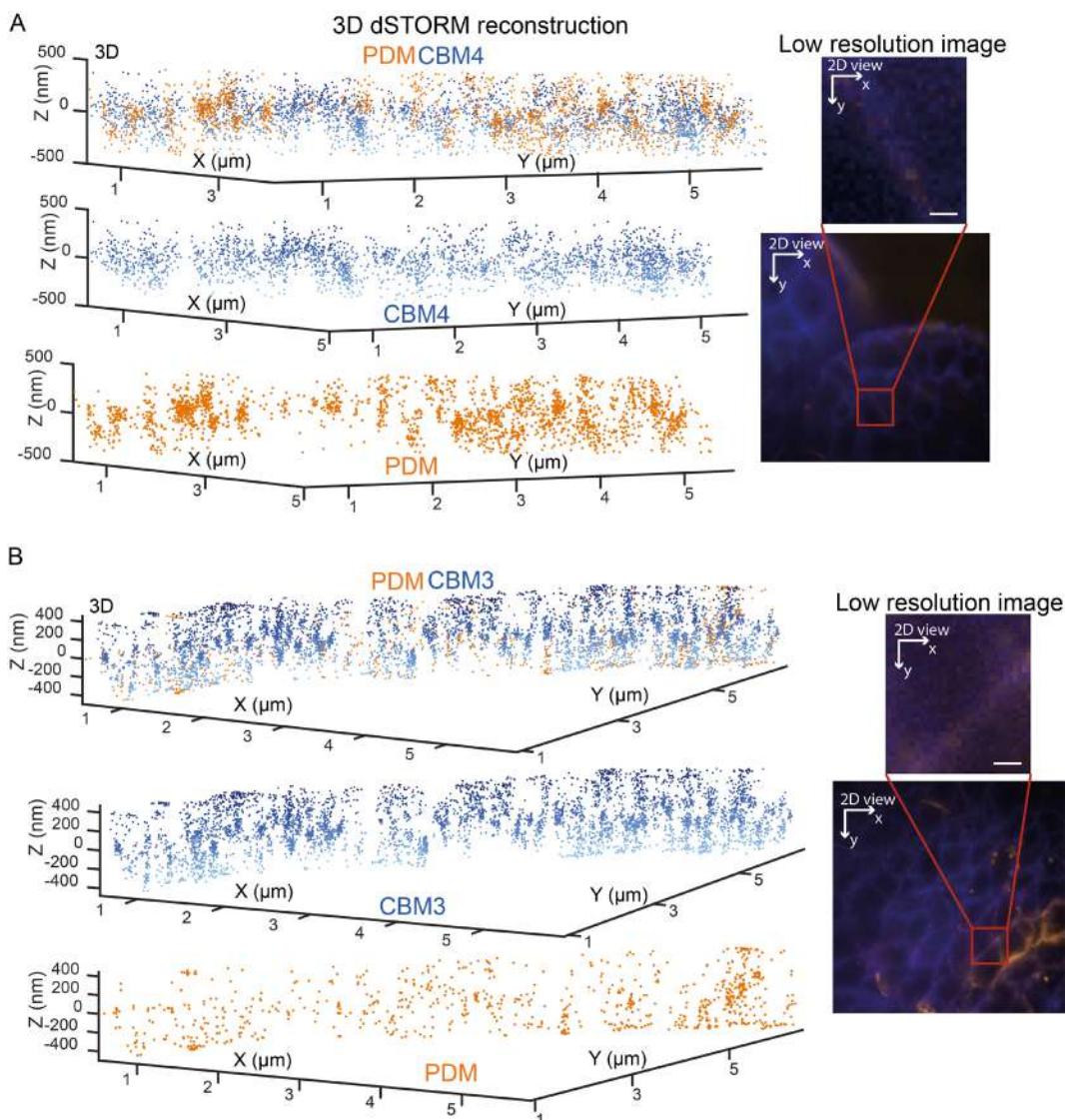


Figure 4. Two-color dSTORM imaging of cellulose (CBM3/4) and (hetero), mannan (PDM) in the *Arabidopsis* primordia, and meristem. Pectins were enzymatically extracted with pectolyase treatment prior to the immunostaining protocol (Step C1). Two-color scatter plots showing the 3D coordinates of localized (A) CBM4 (blue) and PDM (orange) in a meristem, and (B) CBM3 (blue) and PDM (orange) epitopes in primordia. Insets on the left of the plots show a low-resolution fluorescence image with a red square outlining regions presented in the scatter plots. Scale bar, 1 μm. Data were visualized using GrafeoV.2.

Conclusion

IHC is a powerful tool to study subcellular changes in cell wall chemistry. However, it has several limitations. The number of epitopes that could be explored simultaneously is limited (< 4) due to spectral cross-talk and the number of the antibody species. In addition, only epitopes with well-defined antibodies can be detected. Furthermore, the accessibility of the antibody to the inner cell wall components is limited, except if one of the cell wall components is degraded to help penetrability.

Also, the sample preparation for IHC may cause tissue distortion; therefore, spatial scale

measurements and topology may not be absolute. Despite this, multicolor IHC coupled with a novel optical nanoscopy offers unprecedented insights into the complex spatial organization of biomolecules. In combination with super-resolution imaging, multicolor IHC is now changing our vision on the cell wall architecture and plant growth. The cell wall mediates plant cell interactions with the environment, and therefore we expect this technique will help us understand plant immunity, fruit ripening, plant-microbe interaction, plant growth, and yield. Constant improvements in fluorescence probes, single-molecule techniques, and data analysis make that 3D dSTORM has huge potential to refine our knowledge on molecular assemblies and their function (Klein *et al.*, 2014; Kim *et al.*, 2019; Gwosch *et al.*, 2020; Zhang *et al.*, 2020). Hence, “Seeing, contrary to popular wisdom, isn't believing. It's where belief stops, because it isn't needed any more”, Terry Pratchett, Pyramids.

Recipes

1. 2F4 Buffer (T/Ca/S buffer final concentration)

To dilute all the antibodies, prepare blocking buffer and washing buffer (http://www.plantprobes.net/pp_2F4.pdf).

Tris-HCl 20 mM pH 8.2

CaCl₂ 0.5 mM

NaCl 150 mM

The final pH should be 8.0

2. Formaldehyde Alcohol Acetic Acid FAA solution

50% ethanol

10% acetic acid

3.7% formaldehyde

The percentage indicates the final concentration in FAA solution

3. 1 M ammonium chloride solution diluted in 2F4 buffer

Mix 53.489 g of ammonium chloride powder in 1 L of 2F4 buffer

Adjust the pH to 8.0

4. Formaldehyde diluted in 10x 2F4 buffer

Mix 1 volume of 37% formaldehyde solution, 1 volume of 10x 2F4 solution, and 8 volumes of distilled water.

Perform under the fume hood

5. Citrate-Phosphate Buffer for Pectolyase incubation (pH 4.8, Stoll and Blanchard, 1990)

a. 0.2 M Na₂HPO₄·7H₂O

Dissolve 53.65 g in MilliQ water and make 1 L of solution A

b. 0.1 M citric acid

Dissolve 19.21 g in MilliQ water and make 1 L of solution

c. Mix 24.8 ml of solution A and 25.2 ml of solution B and dilute to a total of 100 ml for pH 4.8

If you need, check pH value

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Competing interests

The authors declare no competing interests.

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Dual sgRNA-based Targeted Deletion of Large Genomic Regions and Isolation of Heritable Cas9-free Mutants in *Arabidopsis*

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[Abstract] CR/SPR/Cas9 system directed by a gene-specific single guide RNA (sgRNA) is an effective tool for genome editing such as deletions of few bases in coding genes. However, targeted deletion of larger regions generate loss-of-function alleles that offer a straightforward starting point for functional dissections of genomic loci. We present an easy-to-use strategy including a fast cloning dual-sgRNA vector linked to efficient isolation of heritable Cas9-free genomic deletions to rapidly and cost-effectively generate a targeted heritable genome deletion. This step-by-step protocol includes gRNA design, cloning strategy and mutation detection for *Arabidopsis* and may be adapted for other plant species.

Keywords: CR/SPR/Cas9, Cas9-free, dual-sgRNA, gBlock, Genomic deletion

[Background] Dual sgRNA-directed gene knockout by CR/SPR/Cas9 has been successfully used for genome editing in a variety of organisms (Wang *et al.*, 2013; Chen *et al.*, 2014; Char *et al.*, 2017; Cai *et al.*, 2018; Durr *et al.*, 2018; Cui *et al.*, 2019; Do *et al.*, 2019; Liu *et al.*, 2020). Targeted deletions of genomic DNA regions offer a valuable starting point for functional genomics studies (Hilton and Gersbach, 2015; Ford *et al.*, 2019; Gowthaman *et al.*, 2020). CR/SPR/Cas9-based methods to delete genomic regions benefit from two gRNAs flanking the target DNA regions (Xiao *et al.*, 2013; Canver *et al.*, 2014; Kistler *et al.*, 2015; Song *et al.*, 2016). In plants, one key bottleneck to perform multiplex gene targeting from a single transformation event is to include multiple gBlocks into one binary vector. A gBlock is composed of a RNA Polymerase III (RNAPIII) promoter, a gene specific sgRNA protospacer, a sgRNA scaffold and a RNAPIII terminator. However, gBlock DNA sequences are usually long and repetitive, rendering design expensive for synthetic DNA and laborious by traditional assembly methods (Gao *et al.*, 2016; Peterson *et al.*, 2016; Zhang *et al.*, 2016; Char *et al.*, 2017; Durr *et al.*, 2018; Pauwels *et al.*, 2018; Schuster, 2018; Wu *et al.*, 2018; Hui *et al.*, 2019; Fonseca *et al.*, 2020). For example, Durr *et al.* (2018) developed a dual-sgRNA vector by first modifying a pEN-Chimera entry vector to generate two gBlocks, then inserting two gRNAs into the modified entry vector by restriction enzymes, respectively, and finally cloning two gBlocks into the binary vectors. The multiple steps necessary are laborious and time-consuming. Although multiplex CR/SPR/Cas9 platforms by introducing repetitive gBlocks for targeted genome editing were reported (Ordon *et al.*, 2016; Schuster, 2018), several studies have noted that transformation of highly repetitive DNA sequences can trigger recombination and silencing of the RNA expression cassettes in a variety of species (Ma and Mitra, 2002; Lovett *et al.*, 2004; Brake *et al.*, 2008). To simplify targeted genomic regions and minimize potential recombination in *Arabidopsis*, we

combined and modified existing cloning-based assembly steps. First, we amplified the middle border of our target specific two-gBlocks from a previously developed vector pHEE2E-TRI (Wang *et al.*, 2015) in a single step. Second, we cloned the middle border into a known CRISPR/Cas9 binary vector pKIR1.1 (Maruyama *et al.*, 2013; Tsutsui and Higashiyama, 2017), allowing two-gBlocks with different Poll III-dependent promoters to reduce the repetitiveness. This vector harbors an RPS5A-Cas9 cassette driving high constitutive expression of Cas9 protein at all developmental stages including egg cells, thus achieves highly efficient mutation in the T1 generation of *Arabidopsis*. In addition, the expression cassette OLE1-tagRFP in this system shows red fluorescence in seeds, allowing rapid screening for heritable Cas9-free *Arabidopsis* mutants in the seed of primary transformants. We combined the advantages of the two vectors by a single PCR and a single cloning step, thus providing a simple and reliable protocol to generate stable inherited deletion mutants. Our strategy promises to save cost and time to delete any chromosomal region in *Arabidopsis*, and can be likely adapted for genome editing of multiple genes simultaneously. It also has the potential to simplify genomic deletion in other plant species.

Materials and Reagents

A. Consumables

1. Sterile pipette tips (Axygen, catalog numbers: TF-300-R-S [10 µl], T-350-C-L-R-S [300 µl], TF-1000-R-S [1,000 µl])
2. PCR microtubes (BioExpress, catalog number: T-3135-2)
3. 60 mm x 15 mm round Petri dishes (VWR, catalog number: 100488-404)

B. Competent cells

1. *Escherichia coli* HST08 competent cell (homemade, [protocol 1](#)), store at -80 °C
2. *Agrobacterium tumefaciens* GV3101 competent cells (homemade, [protocol 2](#)), store at -80 °C

C. Vectors

1. pHEE2E-TRI (Addgene, catalog number: 71288), store at -20 °C
2. pKIR1.1 (Addgene, catalog number: 85758), store at -20 °C

Note: Sequences of both vectors can be found in Addgene online.

pHEE2E-TRI sequence: <https://www.addgene.org/71288/sequences/>

pKIR1.1 sequence: <https://www.addgene.org/85758/sequences/>

D. Oligonucleotides 10 µM

Dual-sgRNA1_F: 5'-CACCTGCATACATTGN₂₀(protospacer 1)GTTTTAGAGCTAGAAATAGC-3'

Dual-sgRNA2_R: 5'-CACCTGCATACAAACN₂₀(protospacer 2 reverse complement)CAATCTCTTAGTCGA
CTCTAC-3'

Mlo 1938: 5'-TCCCAGGATTAGAATGATTAGG-3'

Primer_F: 5'-TTCTCTCTTCGCTCTCGTAG-3'
Primer_R: 5'-GGCCCAAATACTCTTTCCAAGAC-3'
Cas9_F: 5'-CAGCCGACAAGAAAGTACAGC-3'
Cas9_R: 5'-ATGGTGGGGTACTTCTCGTG-3'

E. Enzymes and buffers

1. AarI (Thermo Fisher Scientific, catalog number: ER1581), store at -20 °C
2. T4 DNA Ligase (NEB, catalog number: M0202L), store at -20 °C
3. T4 Polynucleotide Kinase (NEB, catalog number: M0201L), store at -20 °C
4. T4 DNA Ligase Reaction Buffer (10x) (NEB, catalog number: B0202S), store at -20 °C
5. Alkaline Phosphatase, Calf Intestinal (CIP) (NEB, catalog number: M0290), store at -20 °C
6. Phusion High-Fidelity DNA Polymerase Kits (New England Biolabs, catalog number: M0530S), store at -20 °C
7. HotMaster Taq DNA Polymerase (VWR, catalog number: QUNT2200330), store at -20 °C
8. Wizard® SV Gel and PCR Clean-Up System (Promega, catalog number: A9282), store at room temperature
9. DNA plasmid kit (VWR, catalog number: D6943-02), store at room temperature

F. Reagents

1. MES (Sigma-Aldrich, catalog number: 4432-31-9), store at room temperature
2. KOH (Sigma-Aldrich, catalog number: 1310-58-3), store at room temperature
3. Sucrose (Sigma-Aldrich, catalog number: 57-50-1), store at room temperature
4. Plant agar (Sigma-Aldrich, catalog number: 9002-18-0), store at room temperature
5. Bacto agar (BD Biosciences, catalog number: 214030), store at room temperature
6. Bacto tryptone (BD Biosciences, catalog number: 211699), store at room temperature
7. Bacto yeast extract (BD Biosciences, catalog number: 212730), store at room temperature
8. Sodium chloride (Fisher Scientific, catalog number: 7647-14-5), store at room temperature
9. Murashige & Skoog medium (Duchefa Biochemie, catalog number: M524), store at 4 °C

G. Antibiotics

1. Spectinomycin (VWR, catalog number: 101454-196), store at -20 °C
2. Rifampicin (VWR, catalog number: 13292-46-1), store in a dry and well-ventilated place
3. Gentamycin (VWR, catalog number: 97062-974), store at 4 °C
4. Kanamycin (VWR, catalog number: 25389-94-09), store at 4 °C

H. Media (see Recipes)

1. LB liquid medium + 100 mg/L spectinomycin (store at 4 °C for one month)
2. LB agar plates + 100 mg/L spectinomycin (store at 4 °C for one month)
3. LB liquid medium + 100 mg/L spectinomycin + 20 mg/L rifampicin + 25 mg/L gentamycin + 25

mg/L kanamycin (store at 4 °C for one month)

4. LB agar plates + 100 mg/L spectinomycin + 20 mg/L rifampicin + 25 mg/L gentamycin + 25 mg/L
5. ½ MS-medium plates

Note: All media need to be autoclaved before adding antibiotics. For plates, 20 ml of media should be used per Petri dish.

Equipment

1. Pipettes (Thermo Scientific, Finnpipette™ F2, catalog numbers: 4642010 [0.2-2 µl], 4642030 [1-10 µl], 4642060 [2-20 µl], 4642080 [20-200 µl], 4642090 [100-1,000 µl])
2. Incubator (Thermo Scientific, catalog number: 51028132)
3. Shakers (Eppendorf, model: New Brunswick™ Innova® 44, catalog number: M1282-0000)
4. Stereo Fluorescence Microscope (Leica, model: M205FA)
5. Gel Doc EQ System (Bio-Rad, Universal Hood II, model: BGDII)
6. Heating blocks (Eppendorf, catalog number: T1317-1EA)
7. ThermoMixer® C (Eppendorf, catalog number: 5382000015)
8. PCR Thermo Cycler (Bio-Rad, model: T100, catalog number: 1861096)
9. Tabletop centrifuge (Thermo Fisher Scientific, catalog number: 75008801)
10. NanoDrop (Thermo Scientific, model: NanoDrop™ 2000C, catalog number: ND2000)
11. Agarose gel electrophoresis equipment (Bio-Rad, catalog number: 1704489EDU)
12. Plant growth chamber (photoperiod: 16 h light/ 8 h dark, temperature: 22 °C in the day/20 °C in the darkness, humidity: 65%, light intensity: 100 µE m⁻² s⁻¹)

Note: No equipment from specific manufacturers is required. Any equivalent device can be used.

Software

1. SnapGene® (SnapGene, <https://www.snapgene.com/>)
2. Image Lab Software (Bio-Rad, <https://www.bio-rad.com/>)

Procedure

O. Overview

The process that generates dual sgRNA-directed large deletion by CRISPR/Cas9 is shown in Figure

1. The vector pKIR1.1 can be ordered from Addgene. A gBlock is composed of a U6 promoter, a gene specific sgRNA protospacer, and a sgRNA scaffold and a terminator.

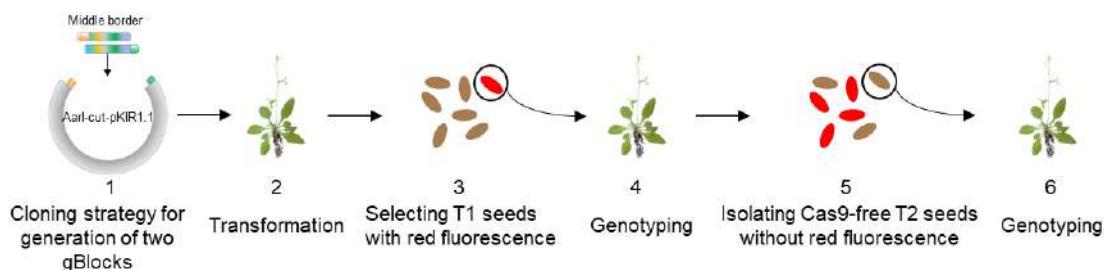


Figure 1. Flowchart for isolation of Cas9-free target mutants. 1. Cloning strategy for generation of two gBlocks. 2. Transformation by floral dipping. 3. Screening T1 seeds with red fluorescence. 4. Genotyping and sequencing T1 plants with red fluorescence to identify candidates and harvest seeds from each individual plant. 5. Isolating Cas9-free T2 seeds without red fluorescence. 6. Genotyping T2 plants to obtain stable and heritable null mutants.

P. Generate the middle border of two-gBlocks

24. Design two gRNAs to target the same gene of interest (GO) with CRISPRdirect (Naito *et al.*, 2015) (Figure 2).

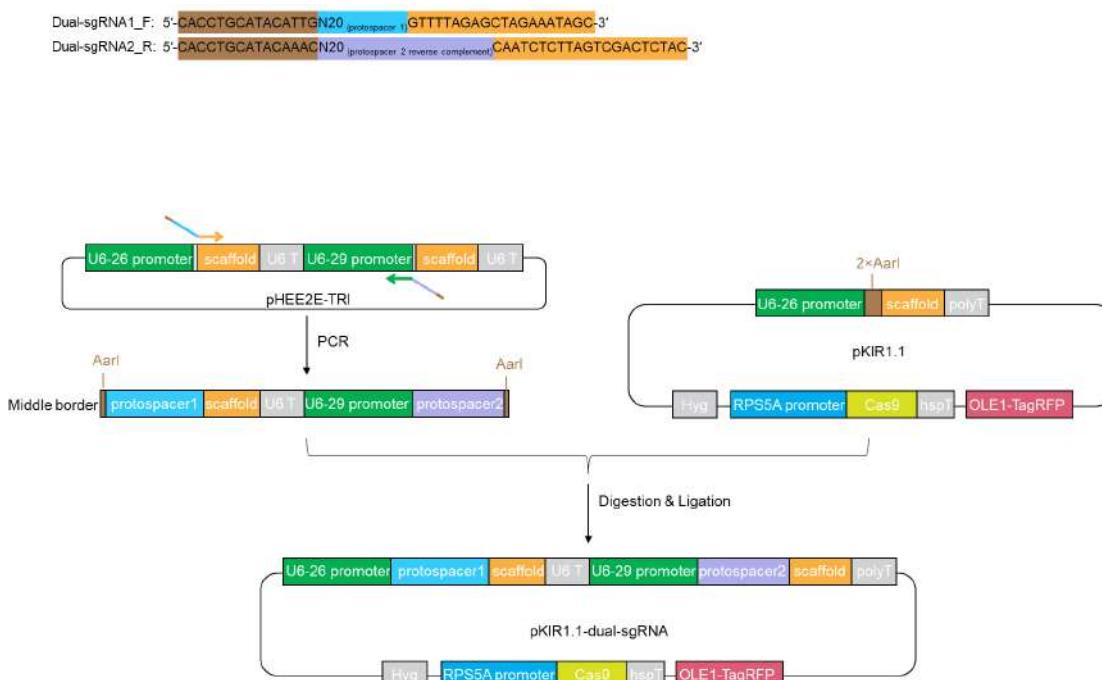


Figure 2. Overview of dual-sgRNAs cloning strategy. Schematic representation of the cloning strategy for the introduction of the middle border of two-gBlocks into the pKIR1.1 backbone. pHEE2E-TRI harbors two sets of gBlocks including gBlock1 (a U6-26 promoter, a 19 bp target sequence 1, a sgRNA scaffold, a terminator) and gBlock2 (a U6-29 promoter, a 19 bp target sequence 2, a sgRNA scaffold, a terminator). Therefore, pHEE2E-TRI can serve as a template to amplify the middle border (AarI-overhang1-protospacer1-scaffold-terminator-U6-29 promoter protospacer2-overhang2-AarI) using a pair of dual-sgRNA primers. After digestion of

pKIR1.1 plasmid and the middle border both by AarI, the middle border can be integrated into the linearized pKIR1.1 backbone to generate pKIR1.1-dual-sgRNA for plant transformation.

- a. Open the CRISPRdirect webpage (<http://crispr.dbcls.jp/>) and paste a target genomic sequence into text field in a FASTA format or a plain nucleotide sequence up to 10 kb.
Note: You can also enter an accession number (e.g., NM_001187) or genome location (e.g., hg19: chr7: 900000-901000) to retrieve sequence, or upload a sequence plain text file in a FASTA format or a plain nucleotide sequence up to 10 kb.
- b. NGG is selected on the ‘PAM sequence requirement’ panel.
- c. Select Thale cress (*Arabidopsis thaliana*) genome, TAIR10 as the organism.
- d. Click on ‘design’.
- e. Select two highlighted protospacer sequence by clicking on ‘show highly specific target only’ as shown in the screen shot below (Figure 3).

Results: [?](#)

Sequence name: NC_003071.7:8122119-8123076 *Arabidopsis thaliana* chromosome 2 sequence
PAM sequence: NGG
Specificity check: Thale cress (*Arabidopsis thaliana*) genome, TAIR10 (Nov, 2010)
Time: 2020-08-01 00:12:09

- Highlighted target positions (e.g., **45 – 67**) indicate sequences that are highly specific and have fewer off-target hits.
- Target sequences with ‘0’ in ‘20mer+PAM’ (in number of target sites column) are shown in gray. Such sequences may possibly span over exon-exon junctions, so avoid using these.
- Target sequences with TTTTs are also shown in gray. Avoid TTTTs in gRNA vectors with pol III promoter.

show **highly specific** target only

Show 20 entries		Search:						
position	target sequence	sequence information				number of target sites ?		
start - end	20mer+PAM (total 23mer)	GC% of 20mer	Tm of 20mer	TTTT in 20mer	restriction sites	20mer +PAM	12mer +PAM	8mer +PAM
103 - 125	- CCG GTGAGTCGCCGTCCCGTTTA [gRNA]	60.00 %	76.77 °C	-		1 [detail]	1 [detail]	44 [detail]
109 - 131	+ AGTCGGCGTCCCGTTAAATT CCG [gRNA]	50.00 %	73.81 °C	-		1 [detail]	1 [detail]	482 [detail]
110 - 132	+ GTCGCCGTCCCGTTAAITC CGG [gRNA]	55.00 %	73.37 °C	-		1 [detail]	1 [detail]	206 [detail]
114 - 136	- CCG TCCCGTTAAATTCGGCTTTC [gRNA]	45.00 %	71.62 °C	-		1 [detail]	1 [detail]	80 [detail]
118 - 140	- CCC GTTTAATTGGGGCTTTCGTC [gRNA]	45.00 %	68.09 °C	-		1 [detail]	1 [detail]	412 [detail]
119 - 141	- CCG TTAAATTGGGGCTTTCGTC [gRNA]	45.00 %	69.60 °C	-		1 [detail]	1 [detail]	281 [detail]
192 - 214	+ GTTGGCTTAACCTTACGGACGT CGG [gRNA]	50.00 %	72.97 °C	-		1 [detail]	1 [detail]	40 [detail]
207 - 229	+ GACGTTGGCCAGCTTCGTA CCG [gRNA]	60.00 %	77.72 °C	-	BsiWI EaeI MscI	1 [detail]	1 [detail]	42 [detail]
215 - 237	- CCA GCTTCGGTACGGCAGCGTG [gRNA]	65.00 %	79.06 °C	-	BsiWI	1 [detail]	1 [detail]	51 [detail]
216 - 238	+ CAGCTTCCGTACGGCAGCGT CCG [gRNA]	65.00 %	80.43 °C	-	BsiWI	1 [detail]	1 [detail]	39 [detail]
217 - 239	+ AGCTTCGGTACGGCAGCGT CGG [gRNA]	60.00 %	79.61 °C	-	BsiWI	1 [detail]	1 [detail]	61 [detail]
222 - 244	- CCG TACGGCCAGCGTGGGCTTAC [gRNA]	60.00 %	79.00 °C	-	BsiWI	1 [detail]	1 [detail]	13 [detail]
236 - 258	+ TGGGCTTACACTAACAGCGT CGG [gRNA]	50.00 %	74.83 °C	-		1 [detail]	1 [detail]	43 [detail]
316 - 338	+ CAGAACTGGTACAGATCTGAAC AGG [gRNA]	45.00 %	66.11 °C	-	BglII	1 [detail]	1 [detail]	242 [detail]

Figure 3. gRNA selection. Example screenshot for gRNA selection using the CRISPRdirect webpage.

*Note: One limitation is to avoid presence of an AarI restriction site on your protospacer sequence. In addition, target sequence can be selected from both DNA strands and should be devoid of TTTTs. The distance between the two sgRNAs depends on your expected deletion regions. Small deletions (<100 bp) can be induced with relatively high frequencies and large one (up to 120 kb) with low frequencies (Ordon et al., 2016) in *Arabidopsis*. The deletion area*

at 5' end or 3' end of the non-coding region doesn't show a frequency difference in this sequence from screen shot (NC_003071.7:8122119-8123076 *Arabidopsis thaliana* chromosome 2 sequence). The 20 nt-protospacer sequence does not have to start with a G, because our linearized plasmids retain a G overhang at the 3' end of the U6 promoter that serves as the first G preferred for initiating transcription at the U6 promoter.

25. Amplify the middle border from the templet pHEE2E-TRI (Figure 2)

- Order forward and reverse primers from idtDNA (<https://www.idtdna.com/>) and dilute them to a final concentration of 10 μ M.

Dual-sgRNA1_F: 5'-CACCTGCATACATTG N_{20} GTAGAGCTAGAAATAGC-3'

Dual-sgRNA2_R: 5'-CACCTGCATACAAAC N_{20} CAATCTCTAGTCGACTCTAC-3'

AarL: CACCTGC

20 nt-protospacer 1 sequence: N_{20}

20 nt-protospacer 2 reverse complement sequence: N_{20}

Overhang1 (ATTG) and overhang2 (AAAC) in yellow

Template specific forward sequence in orange

Template specific reverse sequence in green

Note: Here we take the first and the last gRNA designs in the screen shot as an example:

protospacer 1: TAAACGGGACGGCGACTCAC

protospacer 2: CAGAACATCGTCAGATCTGAAG

protospacer 2 reverse complement sequence: CTTCAGATCTGACGATTCTG

- PCR amplification with the following setup:

5x HF buffer	5 μ l
dNTP (10 mM)	1 μ l
Dual-sgRNA1_F (10 μ M)	1.25 μ l
Dual-sgRNA2_R (10 μ M)	1.25 μ l
pHEE2E-TRI (100 ng/ μ l)	1 μ l
Phusion	0.25 μ l
Water	15.25 μ l
total	25 μl

PCR program:

Initial denaturation	98 °C	30 s	
Denaturation	98 °C	10 s	
Annealing	57 °C	30 s	
Elongation	72 °C	17 s	
Final elongation	72 °C	5 min	
Storage	4 °C	∞	

35 cycles

- c. Run 5 µl PCR product on an agarose gel to check a band with the expected size 620 bp and clean up the rest PCR product using Wizard® SV Gel and PCR Clean-Up System.

Q. Ligate the middle border into pKIR1.1 (Figure 2)

1. Digest and phosphorylate PCR product (the middle border)

- a. Digest the purified PCR product with AarI for 6 hrs at 37 °C.

10x AarI buffer	5.0 µl
Middle border	X µl (=1.5 µg)
AarI	1.5 µl
50x oligo	1.0 µl
Water	42.4 – X µl
Total	50 µl

Note: 50x oligo contains the AarI recognition sequence for stimulating AarI activity (Grigaitė et al., 2002). Both 10x AarI buffer and 50x oligo are supplied with AarI enzyme from Thermo Fisher.

- b. Phosphorylate the digestion mixture for 30 min at 37 °C in the following step, then clean up the mixture using Wizard® SV Gel and PCR Clean-Up System.

Digestion mixture	25 µl
10x T4 ligase buffer	2.83 µl
T4 Polynucleotide Kinase	0.5 µl

Note: Skipping the phosphorylation step will lower the ligation efficiency.

2. Digest and dephosphorylate pKIR1.1

- a. Digest pKIR1.1 with AarI for 6 h at 37 °C.

10x AarI buffer	5.0 µl
pKIR1.1	X µl (= 1.5 µg)
AarI	1.5 µl
50x oligo	1.0 µl
Water	42.4 – X µl
Total	50 µl

- b. Dephosphorylate the pKIR1.1 digestion mixture by 1 µl of phosphatase (CIP) for 30 min at 37 °C and for 10 min at 80 °C.

Digestion mixture	50 μ l
CutSmart® Buffer (10x)	5.67 μ l
CIP	1 μ l

- c. Perform agarose gel electrophoresis with 5 μ l of digestion mixture to test the digest. The expected size of digested pKIR1.1 is around 18.5 kb. Clean up the rest digestion mixture using Wizard® SV Gel and PCR Clean-Up System.

Note: We recommend using pKIR1.1 plasmid as negative control. The digested linear plasmid fragment runs more slowly with a larger band size and can therefore be distinguished from negative control.

3. Ligate the digested middle border with the linearized pKIR1.1 at 16 °C for 30 min to generate the final vector pKIR1.1-dual-sgRNA:

AarI-digested pKIR1.1 vector	X μ l (50 ng)
AarI-digested middle border	X μ l (5 ng)
10x T4 ligase buffer	1.0 μ l
Water	X μ l
T4 Ligase	1.0 μ l
Total	10.0 μl

Note: Here we used vector: insert molar ratio 1:3.

4. Transform *E. coli* competent cells using 10 μ l of ligation product and spread the transformed cells on LB agar plates with 100 mg/L Spectinomycin, then incubate the selection plates overnight at 37 °C (transformation protocol is provided by Clontech: http://www.takara.co.kr/file/manual/pdf/tr_PT5055-2.pdf).

5. Pick 8 colonies to verify the correct insertion (Figure 4) by colony PCR using forward primer Mlo 1938 and your own protospacer reverse primer with the following setup:

10x HotMaster™ Taq Buffer with Mg ²⁺	2 μ l
dNTP (10 mM)	0.4 μ l
Mlo 1938 (10 μ M)	1 μ l
Dual-sgRNA2_R (10 μ M)	1 μ l
HotMaster™ Taq DNA Polymerase	0.1 μ l
Water	15.5 μ l

Pick half of a single colony with a sterile pipette tip and swirl in the PCR reaction.

Use the other half for inoculation.

Total	20 μl
--------------	-----------------------------

PCR program:

Initial denaturation	94 °C	2 min	
Denaturation	94 °C	20 s	
Annealing	57 °C	20 s	35 cycles
Elongation	65 °C	1 min	
Final elongation	65 °C	5 min	
Storage	4 °C		

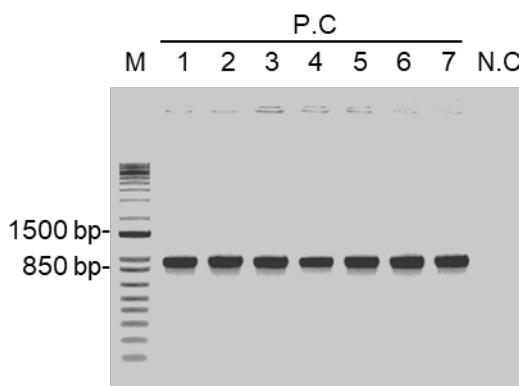


Figure 4. Electrophoresis of the colony-PCR products. The positive colonies show the correct insertion size with 868 bp. P.C: positive colony, N.C: negative colony.

6. Inoculate the positive colony in LB medium with 100 mg/L Spectinomycin and grow overnight.
 7. Extract the plasmid using the DNA plasmid kit.
 8. Sequence the plasmid and verify the sequence of the middle border insertion using primer Mlo 1938.
- R. *Arabidopsis* transformation with pKIR1.1-dual-sgRNA
1. Transform competent *A. tumefaciens* GV3101 cells with 1 µg of pKIR1.1-dual-sgRNA and spread the transformed cells on LB agar plates supplemented with 100 mg/L spectinomycin, 20 mg/L rifampicin, 25 mg/L gentamycin and 25 mg/L kanamycin (Höfgen and Willmitzer, 1988).
 2. After two days of growth at 30 °C, verify plasmid presence in at least three colonies by colony PCR as described before.
 3. Transform *Arabidopsis* plants using *Agrobacterium*-mediated T-DNA transfer with the floral dipping method (Clough and Bent, 1998).
- Note: Per construct, we usually transform at least ten *Arabidopsis* plants. We recommend removing of all present siliques before transformation to avoid excessive screening for transformation events afterwards.*

S. Detect dual sgRNA-directed deletion by Cas9

1. Place the T1 seeds in a glass plate and pick up red fluorescent seeds with a slightly wet

toothpick under a Stereo Fluorescence Microscope using an EL6000 external light source with magnification 10 \times and DsRED filter set (excitation, 546/10 nm; emission, 600/40 nm) (Figure 5).

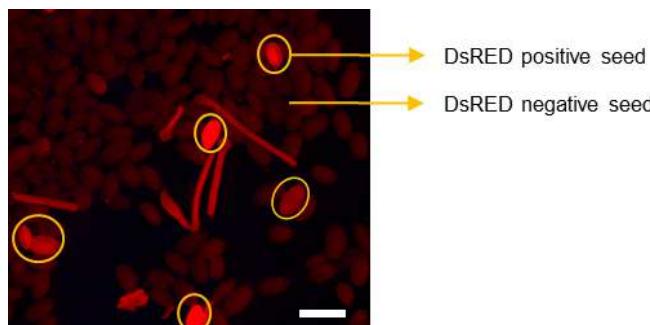


Figure 5. Visual screen for T1 seeds that harbor the CRISPR/Cas9 construct. pKIR1.1 vector can express the s.p. Cas9 protein driven by the RPS5A promoter, that allows high constitutive expression at all developmental stages including the germ cells. pKIR1.1 also contains an expression cassette of OLE1–TagRFP (red fluorescent protein) that exhibits red fluorescence in seeds. Therefore, the transformants containing CRISPR/Cas9 construct can be observed with red fluorescence in T1 seeds as indicated by circles. Scale bar = 1 mm.

Note: The transformation efficiency in this system is around 2.5%, which allows picking up 25 red T1 seeds within 10 min.

- Genotype the targeted deletion of T1 plants observed with red fluorescence in seeds by isolating leaf genomic DNA (gDNA) and subsequent PCR (Edwards *et al.*, 1991) using oligonucleotides flanking the deletion site (\pm 300-500 bp upstream and downstream) (Figure 6):

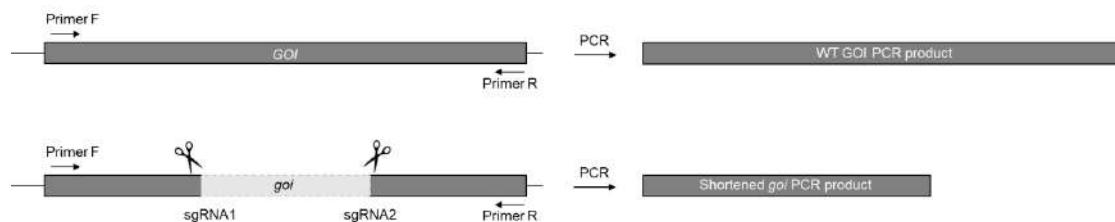


Figure 6. Genotyping the dual-sgRNA induced mutation on GOI. The large deletion of the GOI can be detected by PCR with a pair of primers flanking the deletion site (Primer F and Primer R). A shorter amplicon size can be detected in goi mutant compared to WT.

10x HotMaster™ Taq Buffer with Mg ²⁺	2 μ l
dNTP (10 mM)	0.4 μ l
Primer F (10 μ M)	1 μ l
Primer R (10 μ M)	1 μ l
Leaf gDNA	1 μ l (10 ng)
HotMaster™ Taq DNA Polymerase	0.1 μ l
Water	14.5 μ l

Total **20 µl**

PCR program:

Initial denaturation	94 °C	2 min
Denaturation	94 °C	20 s
Annealing	variable	20 s
Elongation	65 °C	1 min/1 kb
Final elongation	65 °C	5 min
Storage	4 °C	∞

35 cycles

F. Isolate Cas9-Free heritable mutation

1. Harvest T2 seeds from individual genotyped T1 plants for the second round of observation under the Stereo Fluorescence Microscope to select the desired progeny. T2 seeds that do not contain the CRISPR/Cas9 construct can be identified since they lack red fluorescence and isolated.
2. Genotype 10 Cas9-free T2 plants by PCR using oligonucleotides flanking the deletion site using phusion polymerase (Figure 7).

5x HF buffer 5 µl
dNTP (10 mM) 1 µl
Primer F (10 µM) 1.25 µl
Primer R (10 µM) 1.25 µl
Leaf gDNA 1 µl (10 ng)
Phusion 0.25 µl
Water 15.25 µl
Total **25 µl**

PCR program:

Initial denaturation	98 °C	30 s
Denaturation	98 °C	10 s
Annealing	variable	30 s
Elongation	72 °C	30 s/1 kb
Final elongation	72 °C	5 min
Storage	4 °C	∞

35 cycles

3. Separate the PCR products on an agarose gel and extract the shortened *goi* band with the correct size from the gel using the Wizard® SV Gel and PCR Clean-Up System.
4. Sequence PCR product with Sanger sequencing and verify the sequence of the *goi* mutation. Usually heritable deletion events can be identified in plants lacking Cas9 activity after selection of seeds lacking the fluorescent reporter.

Data analysis

The sequencing data can be analyzed by alignment against the wild-type sequence using software SnapGene. Large deletion is generated in *goi* mutant of T2 (Figure 7).

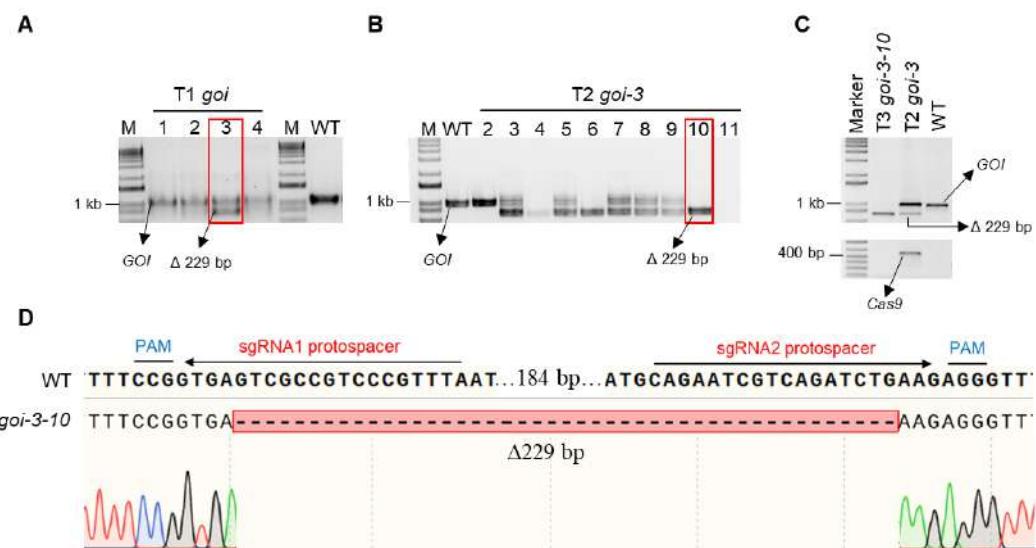


Figure 7. Representative chromatogram of PCR product from *goi* mutant. A. Genotyping of individual T1 plants that display red fluorescence. *goi-3* in red box shows two bands, WT GOI amplicon and *goi* amplicon. This representative *goi* mutant with an expected 229 bp deletion is generated by dual-sgRNA targeting at the genomic region Chr2: 8122228-8122453. PCR product with 1 kb in WT represents no deletion of the GOI. B. In the T2 generation, seeds from T1 *goi-3* are harvested and grown for screening Cas9-free plants. *goi3-10* in red box (without red fluorescence in seeds) shows only the expected size of the *goi* amplicon. C. Bulked seedlings of *goi3-10* in the T3 generation are genotyped. Top, only the expected *goi* amplicon is amplified, suggesting a heritable homozygous deletion. Bottom, no amplification of the Cas9-specific band (406 bp) validates fluorescence-based counter selection of the Cas9 transgene. *goi-3* in the T2 generation (no fluorescence selection) and WT are used as a positive control and negative control, respectively. D. Sequences of the representative deletion fragment of GOI ($\Delta 229$ bp). The alignment is generated with SnapGene. PAM are in blue, sgRNA protospacers are in red, and deleted bases are replaced by a dash in *goi3-10*.

Note: It is mandatory to inspect the sequencing chromatograms carefully and check for the presence of overlapping peaks. Overlapping peaks are a sign of genetic heterogeneity in the sequenced sample.

Recipes

1. LB-liquid medium
10 g/L Bacto-tryptone

- 5 g/L yeast extract
- 5 g/L NaCl
- 2. LB-agar medium
 - 10 g/L Bacto tryptone
 - 5 g/L Bacto yeast extract
 - 5 g/L Sodium chloride (NaCl)
 - 15 g/L Bacto agar for plates
- 3. ½ MS-medium plates
 - 4.4 g/L Murashige & Skoog medium
 - 0.5 g/L MES
 - 10 g/L Sucrose
 - Adjust pH to 5.7 with KOH
 - 10 g/L plant agar for plates

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Competing interests

No competing interests.

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