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Targeting Ultrastructural Events at the Graft Interface of *Arabidopsis thaliana* by A Correlative Light Electron Microscopy Approach

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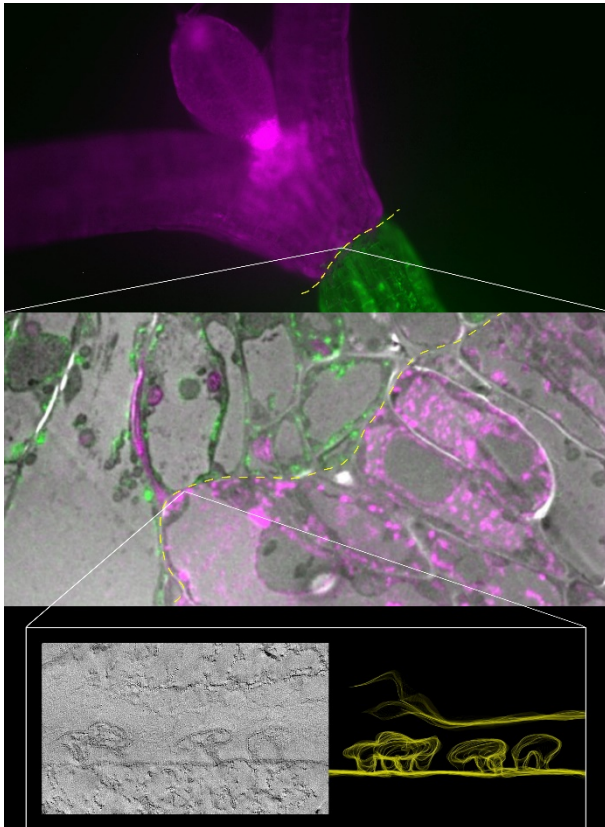
Abstract

Combining two different plants together through grafting is one of the oldest horticultural techniques. In order to survive, both partners must communicate via the formation of de novo connections between the scion and the rootstock. Despite the importance of grafting, the ultrastructural processes occurring at the graft interface remain elusive due to the difficulty of locating the exact interface at the ultrastructural level. To date, only studies with interfamilial grafts showing enough ultrastructural differences were able to reliably localize the grafting interface at the ultrastructural level under electron microscopy. Thanks to the implementation of correlative light electron microscopy (CLEM) approaches where the grafted partners were tagged with fluorescent proteins of different colors, the graft interface was successfully and reliably targeted. Here, we describe a protocol for CLEM for the model plant *Arabidopsis thaliana*, which unambiguously targets the graft interface at the ultrastructural level. Moreover, this protocol is compatible with immunolocalization and electron tomography acquisition to achieve a three-dimensional view of the ultrastructural events of interest in plant tissues.

Keywords: Plant grafting, Correlative microscopy, *Arabidopsis thaliana*, Electron microscopy, Electron tomography

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Graphical abstract



Background

Plant grafting is a process that has been used for centuries in agronomy to combine interesting agronomical traits together, such as resistance and fruit productivity (Mudge, 2009), or to study systemic biological processes, such as epigenetic regulations and long-distance signaling (Molnar et al., 2010; Turnbull, 2010; Turnbull et al., 2002; Wu et al., 2013; Bidadi et al., 2014; Zhang et al., 2014; Yang et al., 2015). Despite its widespread use, the cytologic events required for the establishment of the graft union remain poorly understood (Gautier and Chambaud et al., 2019). During graft union formation, tissues from both partners have to undergo a process of dedifferentiation to form callus cells and set up new pathways of communication such as phloem, xylem, and plasmodesmata. The movement of dyes or fluorescent probes between the scion and the rootstock have been used to resolve the kinetics of vascular connection at the graft interface, but no ultrastructural data are available for the establishment of cell-to-cell connection via plasmodesmata (Melnik et al., 2015; Melnik et al., 2018). Knowledge of the ultrastructural mechanisms underpinning graft union formation is currently very limited because locating precisely the site of contact between the two grafted partners under electron microscopy is challenging due to the difficulty to distinguish the origin/identity of each cell at the graft interface, i.e. to distinguish between cells from the scion and cells from the rootstock (Kollmann et al., 1985; Kollmann and Glockmann, 1985, 1991; Pina et al., 2017; Notaguchi et al., 2020). Attempts were successfully made by Kollmann and Glockmann (1985) and Notaguchi (2020), in which plants from different species showing noticeable ultrastructural differences in the callus cells produced at the graft interface were used.

To overcome the requirement of cytological differences to accurately identify cell origin and properly localize the graft interface, we propose a protocol for correlative light and electron microscopy (CLEM) to unambiguously target the graft interface at the ultrastructural level. At present, the use of CLEM approaches is still scarce for the

analyses of plant tissues (Bell et al., 2013; Marion et al., 2017). Two out of the three CLEM protocols developed for plants are unfortunately not easily compatible with electron tomography due to either the weakness of the embedded resin used (glycol methacrylate) or the absence of resin (Tokuyasu method) (Marion et al., 2017). The protocol described by Bell et al. (2013) presents the advantage of being easily accessible but, because it is based on a chemical fixation, only permits a coarse ultrastructural preservation. Here, in order to combine fluorescence and high ultrastructural resolution in the same sample, we adapted the protocol described by Kukulski et al. (2012a and 2012b), an in-resin fluorescence CLEM method for plant tissues (Kukulski et al., 2012a and 2012b). This protocol allows us to preserve fluorescence and maintain the ultrastructure; further, in combination with electron tomography, this method allows to reach a level of resolution enabling the visualization of the bilayer of the plasma membrane. Moreover, this protocol is compatible with immunogold labeling and is adapted for hypocotyls and roots of plantlets of *Arabidopsis thaliana*. For our study, the hypocotyl of a scion and a rootstock expressing a yellow fluorescent protein (YFP) and monomeric red fluorescent protein (mRFP), respectively, fused to the endoplasmic reticulum-retention motif HDEL, were grafted (Chambaud et al., 2022). By optimizing (1) the grafting procedure, (2) the duration of the freeze-substitution (FS) steps, (3) the thickness of the ultrathin sections, (4) the sample mounting for light microscopy (LM) observation, and (5) the LM acquisition, we were able to observe fluorescence signals and precisely target the graft interface. The compatibility of our CLEM protocol with our previously described electron tomography protocol (Nicolas et al., 2018), allowed to access the three-dimensional (3D) view of ultrastructural elements specifically at the graft interface (Chambaud et al., 2022). Our CLEM protocol has the potential to unravel multiple plant cell processes at the ultrastructural level and was in fact already applied successfully to study autophagy (Gomez et al., 2022) and phase-to-phase separation processes (Dragwidge et al., 2022).

Materials and Reagents

A. Plant culture

1. 1.5 mL Eppendorf tubes (Sarstedt, catalog number: 72.708)
2. 12 N or 37% hydrochloric acid (Carlo Erba, catalog number: 524526)
3. 1.5 mL Eppendorf tube rack (Ratiolab cardboard grid, inserts for cryobox, catalog number: 5020147)
4. Square plastic culture plates for *A. thaliana* seedlings, vertical culture (VWR, catalog number: 391-0444)
5. Bleach (Orapi Europe, catalog number: OR0011)
6. Murashige and Skoog (MS) medium + vitamins (Duchefa Biochemie, catalog number: M0222.0050) for seedlings
7. Plant agar for seedlings culture and grafting (Duchefa Biochemie, catalog number: P1001.1000)
8. Anapore tape (Euromedis, catalog number: 135320) to seal culture plates
9. KOH
10. Bleach solution for seeds sterilization (Recipe 1)
11. Culture medium for seedling and grafting (Recipe 2)
 - a. Half-strength MS medium for seedlings
 - b. Water medium for grafting

B. Transgenic lines

For grafting experiments, we used 35S::HDEL_mRFP and 35S::HDEL_YFP lines, which have strong fluorescent expression (Nelson et al., 2007; Lee et al., 2013). Both lines showed a fluorescence signal that was easily visible through oculars with a zoom microscope with the medium pre-set level for the excitation power and the adapted set filter.

C. Plant grafting

1. Round Petri dishes, 90 × 14.2 mm for grafting (generally used for bacterial culture) (VWR, catalog number 391-0439)
2. Neutral nylon membrane hybond-N (GE Healthcare, catalog number: RPN 203 N)
3. Paraffin film (Bemis, Parafilm 'M') cut into strips of approximately 1 cm wide to seal the Petri dishes

D. High-pressure freezing (HPF)

1. Screw-top 2 mL tubes (Sarstedt, catalog number: 72.693)
2. Toothpicks
3. Bovine serum albumin (BSA) fraction V (Sigma-Aldrich, catalog number: 05482)
4. Methyl cyclohexane (MCH) (Merck Schuchardt OHG 85662 Hohenbrunn, Germany)
5. Distilled water to cut sample
6. Membrane carriers (100 μm deep with 1.5 mm diameter) (Leica Microsystems, catalog numbers: 16707898)
7. 20% BSA solution for cryoprotection during HPF (see Recipe 3)

E. Freeze-substitution (FS)

1. Disposable regular pipettes, 1, 2, and 3 mL (VWR, catalog numbers: 612-2850, 612-2851, and 612-1681) and FS-specific 1 mL pipettes with thin tips (Ratiolab, catalog number: 2600155)
2. Wheaton glass sample vials with snap-cap for cryomix preparation (DWK Life Sciences, WHEATON, catalog number: 225536)
3. Personal cartridge half mask 6100 (Honeywell International, catalog number: 1029471)
4. Aluminum foil
5. Uranyl acetate powder (Merck, catalog number: 8473)
6. Pure methanol (Fisher Scientific, catalog number: M/4062/17)
7. Ultrapure 100% ethanol and acetone (VWR, catalog numbers: 83813.440 and 20066.558, respectively)
8. Colored nail polish (color does not matter)
9. 12.5 mL reagent containers with screw caps (Leica Microsystems, catalog number: 16707158)
10. HM20 resin (Electron Microscopy Sciences, catalog number 14345)
11. Reagent bath (Leica Microsystems, catalog number: 16707154)
12. Plastic mold flow-through rings (Leica Microsystems: 16707157)
13. Cryosubstitution uranyl-acetate stock solution (20%) (see Recipe 4)
14. Cryosubstitution mix (see Recipe 5)
15. HM20 solutions (see Recipe 6)

F. Ultramicrotomy

1. Crystallizing dish (Fisher Scientific, catalog number: 11766582)
2. Fast absorbent paper filters (Whatman, Filter papers 41) (GE Healthcare, catalog number: 1441-070)
3. Glass rods (Fisher Scientific, catalog number: 12441627)
4. Gilder standard hexagonal 200 mesh copper grids (Electron Microscopy Sciences, catalog number: G200HS-Cu) to coat with parlodion or commercial grids with formvar (Electron Microscopy Sciences, catalog number: FCFT200-CU)
5. Razor blades for coarse block preparation (Electron Microscopy Sciences, catalog number: 71990)
6. Pasteur pipet (VWR, catalog number: 612-1720)
7. 1 mL syringe filter (VWR, catalog number: 514-0061)
8. 10 mL syringe (Terumo, catalog number: SS+10ES1)
9. Solid parlodion (Electron Microscopy Sciences, catalog number: 19220)
10. Isoamyl-acetate (Sigma-Aldrich, catalog number: W205532)

11. Toluidine blue powder (Sigma-Aldrich, catalog number: T3260)
12. Sodium borate powder
13. Ultrapure (Milli-Q) water
14. 2% parlodion solution for grid filming (see Recipe 7)
15. Toluidine blue solution (see Recipe 8) for screening block sections on tabletop microscope during resin block milling

G. Electron microscopy

1. Gold colloid 5 nm (BBI Solution, catalog number: EMGC5)
2. Gold fiducials solution (see Recipe 9)

Equipment

A. Plant culture

1. Desiccator used as hermetic chamber
2. Fume hood
3. Glass crystallizing dish
4. Fridge for vernalization
5. -80°C freezer for sterilization

B. Plant grafting

1. Dissecting microscope with a minimal working distance of 5 cm (Leica Microsystems, model: Leica MZFLIII)
2. Glass beaker for sterilization of tools with ethanol
3. Spray for ethanol
4. Dissecting micro-knives 15° 5 mm (Fine Science Tools, catalog number: 72-1551)
5. Precision tweezers [EMS style 5X, catalog number: 78320-5X (soft) and 78520-5X (hard)]

C. Fluorescence screening

1. Zoom microscope (Zeiss Axiozoom.V16) equipped with a 0.5× objective with a numerical aperture of 0.125. The fluorescent part is composed by the fluorescence source Optoprime sola SE II and filter sets (GFP filter set: excitation window between 460 and 480 nm and emission window between 500 and 548 nm; mRFP filter set: excitation window between 540 and 580 nm and emission window up to 593 nm).

D. High-pressure freezing (HPF)

1. Liquid nitrogen, liquid nitrogen container, and adapted personal protection gear
2. Air compressor (JUN-AIR)
3. Leica EM-PACT1 machine (Leica Microsystems)
4. Leica loading system
5. Pod holders (Leica, catalog number: 16706832)
6. Pods (Leica, catalog number: 16706838)
7. Regular biomolecular pipettes, ranging from 2 to 1,000 µL
8. Binocular with transmission illumination for root dissection (Nikon, model: SMZ-10A)
9. Heating surface for quick drying of pods and pod holders during the session
10. Insulated tweezers for manipulation in liquid nitrogen (VOMM Germany, 22 SA ESD)

11. Metal containers for frozen sample transfer and associated screwdriver for lifting procedures (provided with EMPACT1)
12. Torque screwdriver (TOHNICHI, Torque driver RTD60CN) set to 30 N.cm

E. Freeze-substitution (FS)

1. Leica automatic freeze-substitution AFS2 (Leica Microsystems, model: Leica AFS2)
2. Leica freeze-substitution processor FSP (Leica Microsystems, model: Leica FSP)
3. Metal socket for mold containers
4. Microneedles for separating the membrane carriers from frozen samples [microneedle 0.025 mm (Electron Microscopy Science, catalog number: 62091-01) and 5 μ m (Ted Pella, catalog number: 13625)]
5. Ventilated fume hood

F. Ultramicrotomy

1. Block holders
2. Diamond trimming knife for first milling steps to reach the sample (Drukker 8 mm Histoknife Dry, discontinued)
3. Diamond knives Trim20 dry (Diatome, catalog number: DTB20), Ultra wet 45° (Diatome, catalog number: DU4530), and Histo wet (Diatome, catalog number: DH4560)
4. ELMO glow discharger (optional) (CORDOUAN Technologies)
5. Grid carriers for grid storage
6. Leica Ultracut UC7 (Leica Microsystems, model: Leica Ultracut UC7)
7. Tabletop microscope (Olympus, model: CX-41)
8. Precision weight scale for parlodion weighting (Sartorius, model: TE124S)

G. Confocal microscopy

1. Sensitive confocal microscope (Zeiss Microscopy, model: Zeiss LSM 880) and 63 \times NA 1.4 oil immersion objective

H. Electron microscopy

1. FEI Tecnai G2 Spirit 120 kV equipped with Eagle 4K \times 4K high sensibility CCD camera
2. Advanced tomography holder (Fischione instruments, model: 2020)

Software

1. Zeiss Zen Dark version 2.3 software (<https://www.zeiss.com/microscopy/int/products/microscope-software/zen.html>) used for confocal acquisition
2. Tecnai imaging and analysis (TIA) version 4.5 software was used in conjunction with transmission electron microscopy (TEM) User Interface software version 4.5 to control and acquire micrograph with the electron microscopy (<https://www.fei.com/software/>)
3. Xplore3D version 4.5 (FEI) was used for automated tilt series acquisitions (<https://www.fei.com/software/>)
4. ImageJ version 1.53n (<https://imagej.nih.gov/ij/download.html>) with the Bio-Format version 6.9 plugin (<https://www.openmicroscopy.org/bio-formats/>) to open and process the Zeiss .czi files and to open the .mrc tilt series files and tomograms.
5. IMOD suite version 4.11.6 (<http://bio3d.colorado.edu/imod/>). All processing of tomograms was done using the IMOD suite (Kremer et al., 1996), from the alignment of the tilt series to tomogram reconstruction

6. For visualization purposes only, Adobe Photoshop version 23.0.1 is used for TEM mosaic assembling using photomerge function.
7. ICY version 2.4.2.0 and its plugin ec-CLEM version 1.0.1.5 (<https://icy.bioimageanalysis.org/plugin/ec-clem/>) were used for LM and TEM correlation

Procedure

A. Plant grafting

The *A. thaliana* hypocotyl grafting procedure was adapted and optimized from the *Two Segment Shoot-Root Graft* part of the protocol of Melnyk (2017a). Finding the good level of humidity is the most challenging part of Melnyk's protocol. In our hands, replacing the water-soaked Whatman paper by a water-based agar medium was key to maintain constant humidity for several days after grafting and increased our grafting success from 50% to 80%. To target the graft interface with CLEM, we grafted transgenic lines expressing fluorescent proteins fused to the endoplasmic reticulum-retention motif HDEL (35S::HDEL-YFP and 35S::HDEL-mRFP1.2) (Dean and Pelham, 1990; Knox et al., 2015; Chambaud et al., 2022). The constitutive 35S promoter was used to ensure a strong initial fluorescent signal.

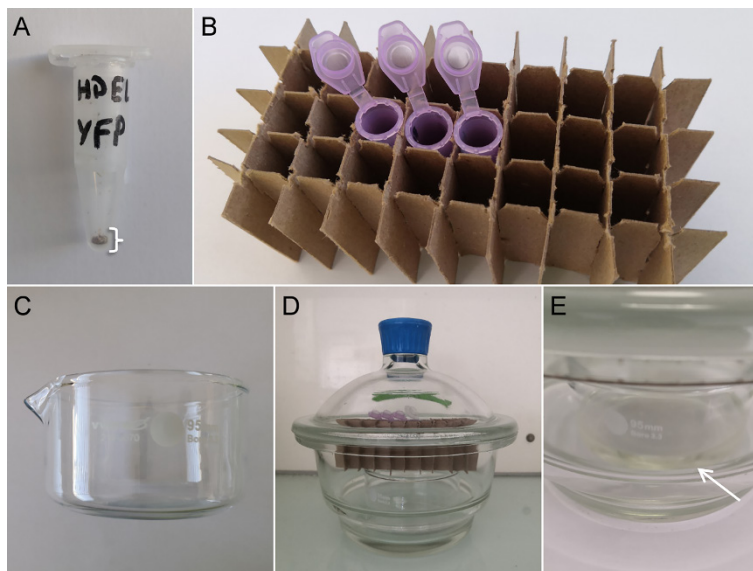


Figure 1. Seed sterilization procedure. (A) Appropriate number of seeds in a 1.5 mL sample tube for a proper sterilization. (B) For gas sterilization, place them open in a tube rack. (C) Glass crystallizing dish for the bleach. (D) Hermetic chamber with bleach in the glass crystallizing dish, tube rack, and open Eppendorf tube containing seeds. (E) When mixing bleach with hydrochloric acid, the solution becomes yellow (white arrow) and a faint yellow haze of chloride gas is visible.

1. Plant culture

- a. Sterilize *A. thaliana* seeds with chlorine gas (adapted from Lindsey et al., 2017).
 - i. Aliquot a small number of seeds (approximately 100–150) into a 1.5 mL centrifuge tube labeled with a permanent pen (covered in adhesive tape to avoid washing off of the ink), close the caps, and place them for 20 min in a -80°C freezer (Figure 1A).
 - ii. The sterilization will take place in a hermetic chamber, typically a glass desiccator, under a fume hood. In the desiccator, place a small glass crystallizing dish containing 50 mL of bleach solution (see Recipe 1) (Figure 1C and 1D).

- iii. Uncap centrifuge tubes before placing them in a tube rack in the chamber. Add 1.5 mL of 12 N hydrochloric acid to the bleach solution that is in the crystallizing dish without mixing and quickly close the chamber. The yellow haze of chlorine gas should be visible at this point (Figure 1E). Let the seeds in the chamber from 4 h to overnight (e.g., 6 pm–9 am).
- iv. Open the chamber and close the centrifuge tubes. Leave the chamber open under the fume hood for one day until the reaction between hydrochloric acid and bleach ends. The reaction produces chlorine gas, sodium chloride, and water. You can then discard the solution. Note that if the seed germination seems affected, the incubation time should be reduced to 1–3 h.
- b. Under a laminar flow hood, gently sprinkle the seeds over square Petri dishes containing half-strength MS medium with vitamins. Thanks to chloride gas sterilization, homogeneous distribution of the dry seeds is easily achieved. Seal the plate with anapore tape.
- c. For vernalization and stratification, place the plates horizontally with the seeds on the upper face at 4°C in the dark for 2–5 d maximum.
- d. Transfer the plates to a growth chamber regulated at 20–23°C with photosynthetic photon flux density (PPFD) of 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of light. Put the plates vertically so that the hypocotyls grow as straight as possible. Note that short-day growing conditions (such as 8 h of light per day) give rise to more elongated and thinner hypocotyls, which facilitate the grafting procedure and microscopy experiments (Melnyk, 2017a, 2017b). Once the two first true leaves are just visible under the binocular microscope, grafting can be done (Figure 2). In general, seedlings grown under short or long days (i.e., 8 and 16 h of light) have to be 5 or 7 days old, respectively, for grafting. At all times, you need to monitor plant growth to graft at the more appropriate time.

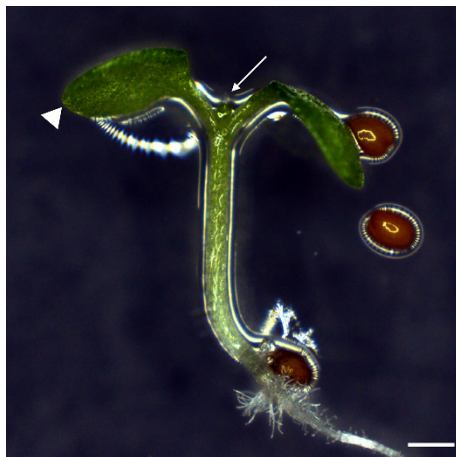


Figure 2. The ideal Arabidopsis plantlets for grafting. An ideal *A. thaliana* seedling for grafting has a straight hypocotyl, very small first leaves (white arrow), and cotyledons that should not bend over the hypocotyl (white arrowheads). Scale bar: 500 μm .

2. Grafting

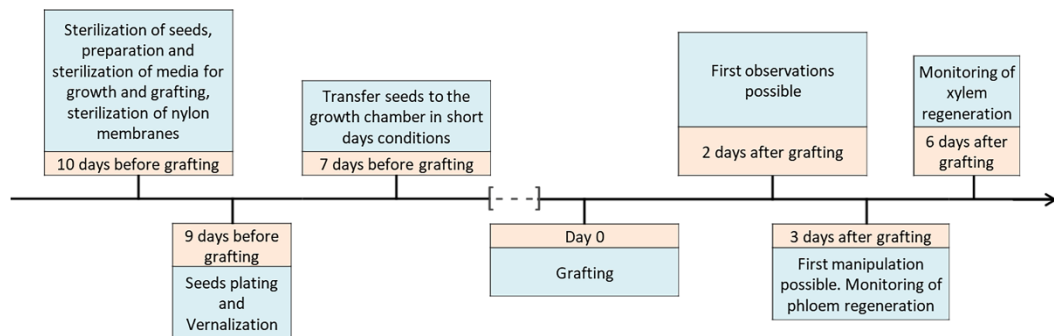


Figure 3. Workflow of grafting experiments

The absence of sucrose in the culture and grafting media makes it possible to graft in non-sterile conditions if you do not plan to keep the grafts in vitro for more than six days. In our grafting studies, we grafted on the bench in non-sterile conditions because we worked on samples at three and six days after grafting. Nevertheless, we work on a 70% ethanol pre-cleaned bench with sterilized tools to limit contamination. The dissecting microscope used must have a working distance (distance between the lens and its focal plane) of at least 5 cm to allow enough space for the manipulations. We usually use a magnification between 6× and 20× with annular illumination or optical fiber systems. Performing grafting is tedious and requires precise and quick manipulations. Try to avoid coffee and other stimulants before attempting grafting experiments. With practice, the grafting technique allows you to reach a success rate of 80% to 90% with a rate of 70 grafts per hour. Following the workflow of grafting experiments proposed (Figure 3) can help you to organize your schedule. For the grafting day, book the epifluorescence zoom microscope and the stereomicroscope and prepare the material (Figure 4).

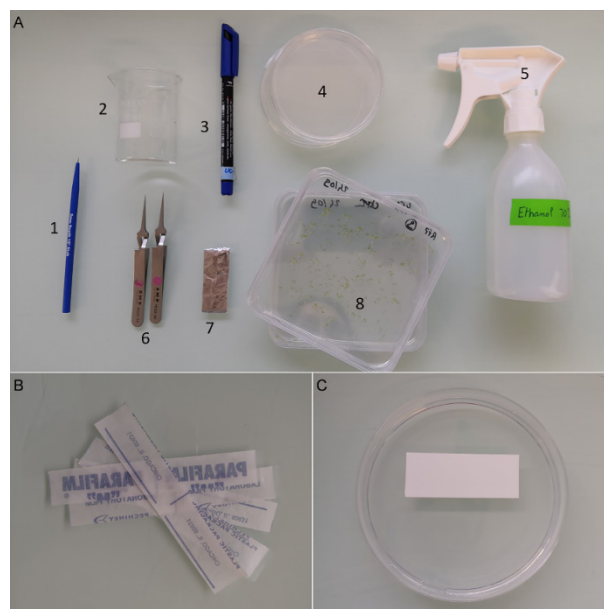
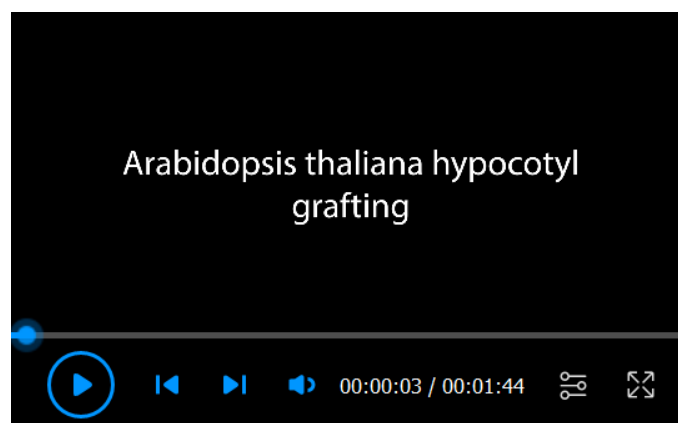


Figure 4. Grafting tools. (A) 1. Dissecting micro-knife for grafting; 2. Glass beaker for sterilization of tools; 3. Permanent marker to label Petri dishes; 4. Round Petri dishes for grafting; 5. Spray with ethanol for tools and bench cleaning; 6. Fine tweezers; 7. Sterile nylon membrane wrapped in aluminum foil; 8. Plants to graft. (B) Parafilm strips to seal Petri dishes after grafting. (C) Positioning of nylon membrane prior to grafting.

- a. Before grafting
 - i. Cut a nylon membrane in 2×5 cm pieces and wrap them in aluminum foil.
 - ii. Prepare the grafting medium with 1.6% plant agar in distilled water and buffer it at pH 5.8 with 1 N KOH.
 - iii. Autoclave the grafting medium and the pieces of nylon membranes wrapped in aluminum foil at 120°C for 20 min.
 - iv. When the grafting medium is handleable (avoid burns after autoclaving), fill the round Petri dishes with approximately 20 mL of agar under a laminar flow hood.
- b. For grafting step
 - i. Using a fluorescence zoom microscope with an excitation power set at the medium pre-set level, identify plantlets showing a strong expression of fluorescence with excitation power.
 - ii. Gather Petri dishes, micro-knives, tweezers, nylon membranes, permanent pen, and strips of parafilm (Figure 4).
 - iii. Clean the dissecting microscope, micro-knives, and tweezers by spraying 70% ethanol and let them dry completely before usage. If you have to graft more than two plates, repeat the cleaning step every two plates.
 - iv. Label your Petri dishes with the plant lines you will use as the scion and the rootstock.
 - v. Put a piece of nylon membrane on the upper part of each box (Figure 4C).
- c. Among fluorescent plantlets, select the ones with homogeneous size, hypocotyls as straight as possible, and cotyledons not bent over hypocotyls (Figure 2). Put them in a row on the membrane (Figure 5). To move them, you can use tweezers or a toothpick that does not pinch the tissue. Note that you have to avoid any damage to the hypocotyl, shoot, and root. If you want to use the two genotypes as scion or rootstock, you could put six plants of the first genotype in the same row of six plants of the second genotype (Figure 5A). If you wish to use a genotype only as scion and the other only as rootstock, you could dispose the plants in two rows of 12 plants (Figure 5B).
- d. The membrane is used as a cutting surface. Cut on it to avoid damages to the micro-knife.

Notes:

 - i. *If the micro-knife is used properly, each can be used for approximately 400–500 grafts. It must permit a sharp sectioning in only one movement. Under a cleaned dissecting microscope, cut off and discard one cotyledon (choose the smallest, most damaged cotyledon that is bent over the hypocotyl) to allow the seedling to lie flat on the membrane (Figure 6A, B, C). Cut transversally through the hypocotyl, just under the cotyledons, without crushing the plant (Figure 6D).*
 - ii. *Cutting the hypocotyl too low could impair the grafting success due to the formation of adventitious root from the scion (Video 1).*



Video 1. Arabidopsis thaliana hypocotyl grafting

- e. Lift the scion by the petiole with closed tweezers and place it against the rootstock of another plant (Figure 6F and 6G). Most of the surfaces of both sectioned parts have to be in contact. You can improve the positioning by gently pushing the cut scion against the cut rootstock with the closed tweezers to bring both partners in direct contact (Figure 6G and 6H).

Note: We recommend that you graft together hypocotyls with similar diameters and avoid moving the cut rootstock, which is very fragile. The moisture level is crucial during graft union formation. Grafting onto grafting medium (water with 1.6% agar) keeps the moisture level constant and allows to keep the grafts in vitro six days after grafting.

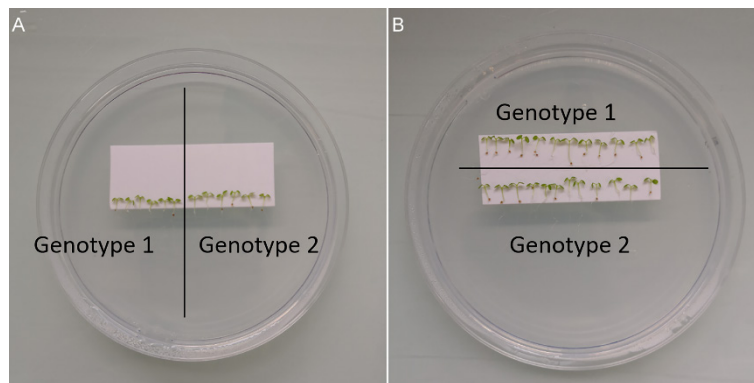


Figure 5. Grafting preparation. (A) When wishing to keep both directions of grafting (genotype 1/genotype 2 and genotype 2/genotype 1), a 6/6 graft scheme can be used. Scions and rootstocks will be prepared, cut, and exchanged between the two genotypes. (B) When a single direction of grafting is required (genotype 1/genotype 2 as above), a 12/12 graft scheme can be used. Bring the scions on the top row (genotype 1) before cutting and discarding their roots. The bottom row (genotype 2) contains rootstocks. Their apical part will be cut and discarded. Scions prepared from genotype 1 will be moved and grafted to rootstocks from genotype 2.

- f. Repeat steps c., d., and e. until all the plants are grafted. To save time, you can do this in a row: (1) cut and discard one cotyledon on each seedling, (2) cut all the shoots, (3) move them next to the prepared rootstocks, (4) align and push the scions onto the rootstocks.

When the grafting is done, be very cautious with the plates to avoid sudden movements that could induce the scion falling off the rootstock. Close the lid and seal the plates with a Parafilm strip. You can label the plate with the time you ended grafting so that you know the age of the graft. Carefully put the plate vertically back in the growth chamber at 20–23°C in PPFD of 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light in either short- or long-day conditions. Note: If you grafted properly, you can begin to manipulate your graft (i.e., observing it under a microscope, fix tissues, transferring it to a new medium, etc.) 48 h after grafting. After 3 d, the grafts become slightly more robust as phloem should be connected. After 6–7 d, xylem is connected, and the grafted plant becomes quite robust. When beginning grafting experiments, you may need to assess your grafting success rate at first. You can rely on several criteria to evaluate grafting success (Melnyk, 2017b), such as the resumption of root growth, usually with emergence of lateral roots, and the absence of adventitious root formation in the scion. You can also monitor the phloem connection by grafting scions expressing pSUC2::GFP [i.e., green fluorescent protein (GFP) under the phloem-specific SUC2 promoter] and observing the movement of GFP to the rootstock, or by applying carboxyfluorescein diacetate (CFDA) to the scion or rootstock to evaluate phloem and xylem connectivity as described in Melnyk (2017b).

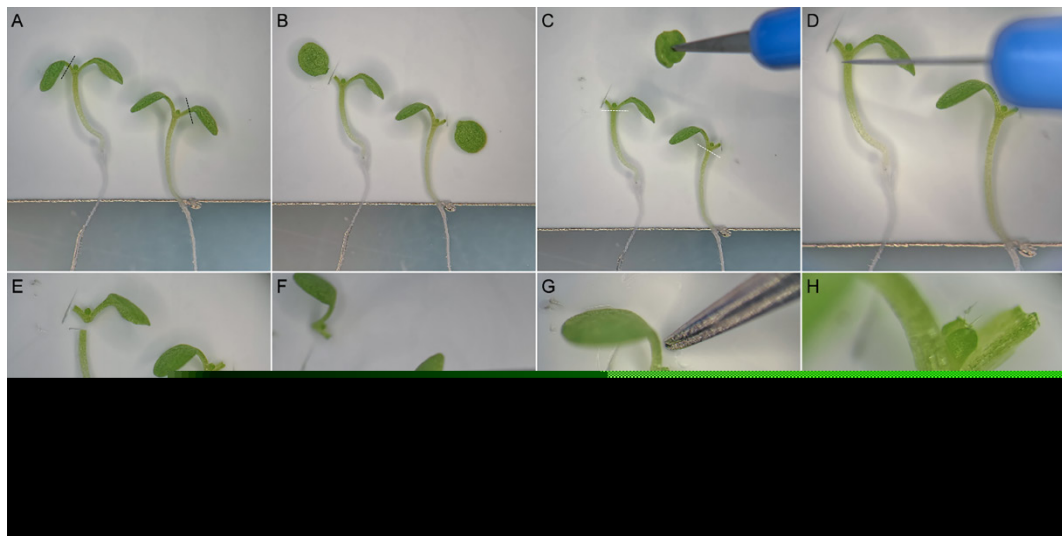


Figure 6. The step-by-step procedure of grafting. The two plants are placed side by side on a nylon membrane and one cotyledon of each plant is cut along the black dashed lines (**A and B**) and then discarded (**C**). Hypocotyls are cut just below the petiole of cotyledons along the white dashed lines (**D and E**). Scions are exchanged between plants by sticking them to the tip of the tweezers (**F**). Scion is carefully brought close to the rootstock with the tip of the tweezers (**G**). When properly grafted, the graft junction (black arrowhead) is barely visible (**H**).

B. CLEM

Before fixation, ensure that the fluorescence signals are easily visible through the oculars for individual samples with non-destructive observations (stereomicroscopy or macroscopy).

1. HPF

Note: HPF was done using the Leica EM-PACT1 machine (Studer et al., 2001). A detailed protocol is described in Nicolas et al. (2018). This procedure involves the manipulation of liquid nitrogen; if you are not familiar with its handling, please refer to the official guidelines. Moreover, we recommend fixing approximately 10 samples by condition and to process all your samples of one condition as a batch, before fixing the next one. It will help you avoid mixing up the different grafts. Moreover, we recommend starting the HPF and FS either on Monday or Tuesday to finish handling for block preparation on the next Thursday or Friday (Table 2).

- a. Prepare the EM-PACT1 machine by (1) filling its dewar with liquid nitrogen, (2) filling the MCH chamber, and (3) evacuating the air out the circuit by two blank shoots.
- b. Charge the membrane carrier (100 μm deep carriers) on the Leica loading system.
- c. Pick a grafted plant out of the Petri dish and place it on a glass slide covered with water under a binocular zoom microscope.

Note: The following steps should be carried out as fast as possible to limit biological responses from the cutting and BSA drying.
- d. Fill the carrier with 20% BSA in water with a 0.5–2 μL pipette until the liquid surface is domed in the well of the carrier. Ensure that there are no air bubbles.
- e. Under the binocular zoom microscope, dissect the graft interface in water on a glass slide with a razor blade and then carefully pick it up with a toothpick.
- f. Dispose the graft interface into the BSA in the membrane carrier before pushing the membrane carrier in the pod using the Leica loading system.
- g. Close the pod using the Torque screwdriver (set on 30 N.cm) and remove the Leica loading system.
- h. Load the sample in the EM-PACT1 machine and shoot.

