Chapter 7

Live Imaging of *Arabidopsis* Axillary Meristems

Bihai Shi, Hongli Wang, and Yuling Jiao

Abstract

Axillary meristems (AMs) are established postembryonically at the leaf axils and can develop into lateral branches. The initiation of AMs establishes new growth axis and is of primary importance for understanding plant development. Understanding plant development requires live imaging of morphogenesis and gene expression. However, AMs are embedded in the leaf axil, making it challenging to perform live imaging. In this chapter, we describe how to prepare and culture *Arabidopsis thaliana* leaves in vitro, to perform one-time or time-lapse imaging of AM initiation with a confocal microscope.

Key words Axillary meristem, In vitro culture, Confocal, Live imaging, *Arabidopsis*

1 Introduction

Plants continuously form new tissues and organs throughout their life span in a modular manner. Each module, termed as phytomer, includes a leaf, an axillary meristem (AM) at the leaf axil, and a segment of internode. AMs form lateral buds and have the potential to further develop into lateral branches. The activity of AMs thus determines the plant architecture and crop yield [1].

Development is a dynamic progress involving cell division, spatiotemporal gene expression, and protein localization. Traditional techniques, such as in situ hybridization and analysis of transgenic plants harboring nonfluorescent molecular markers, use dead, fixed samples and lack proper spatiotemporal resolution. The development of confocal live-imaging technique opens a new way to investigate developmental processes, such as AM initiation in live samples at the cellular resolution. In this chapter, we present a detailed live-imaging method to study AM initiation using an upright confocal microscope. This method has been used to capture the expression dynamics of the auxin sensor DII-Venus and meristem marker genes during AM initiation [2–4]. This chapter also covers preparation and in vitro culture of leaves for imaging, including important points and tips.

Muhammad Naseem and Thomas Dandekar (eds.), *Plant Stem Cells: Methods and Protocols*, Methods in Molecular Biology, vol. 2094, https://doi.org/10.1007/978-1-0716-0183-9_7,

2 Materials

1. Confocal laser scanning microscope (CLSM): An upright confocal microscope is preferred, but an inverted microscope could also be used [5, 6].
2. Water dipping lens: 40× objective lens with a large working distance (e.g., Nikon NIR Apo 40×, 0.8 NA, 3.5 mm working distance).
3. Stereomicroscope: For tissue dissection, a magnification of at least 50× is suggested.
4. Tweezers sterilizer (e.g., Keller Steri 250 dry beads sterilizer; Keller, Swiss) (*see* **Note 1**).
5. Laminar flow hood: For time-lapse imaging, all the steps mentioned in the Methods section before confocal imaging are performed in a hood to avoid contamination.
6. Tweezers: Tweezers with fine tips are used to transfer and dissect seedlings and leaves.
7. Hypodermic sterile insulin syringes: Sterile syringe needle tips act as sharp blades to cut leaves from the seedling.
8. Murashige and Skoog (MS) medium: 1/2× MS basal salt mixture with vitamins, 2% sucrose, pH = 5.8, 0.8% agarose, autoclaved at 121 °C for 20 min.
9. MS plates for seed germination: Round glass petri dishes (~10 cm in diameter and ~2 cm in depth) filled with ~0.7 cm MS medium in depth.
10. In vitro culture medium: 1× MS basal salt mixture with vitamins, 2% sucrose, 0.0005% (w/w) folic acid, 0.01% (w/w) myo-inositol, pH = 6.0, 0.3% phytagel, autoclaved at 113 °C for 15 min (*see* **Note 2**).
11. Dissecting plates: Round glass petri dishes (~10 cm in diameter and ~2 cm in depth) filled with autoclaved 3% agarose (*see* **Note 3**).
12. Imaging plates: For time-lapse imaging, use round plastic petri dishes (~6 cm in diameter and ~1.5 cm in depth; Corning Life Sciences, Corning, NY, USA) with ~0.7 cm MS medium in depth. After cooling down, top up with a layer (~0.3 cm in depth) of autoclaved 1% agarose to minimize contamination. For one-time imaging, use dishes with 1 cm 1% agarose instead.
13. In vitro culturing plates: Round glass petri dishes with ~1 cm in depth in vitro culture medium.
14. Plants for imaging: 15-d-old *Arabidopsis thaliana* seedlings grown on MS plates under short-day conditions (8-h light at

21 °C, and 16-h dark at 19 °C) (*see* **Note 4**). We have used L*er*, Col-0 and Ws, and obtained comparable results. We expect other ecotypes to work as well.

15. FM4–64 solution: For cell membrane staining (Thermo Fisher Scientific, Waltham, MA, USA; stock concentration, 20 mg/mL; working concentration, 50 μg/mL) [7].
16. Illumination incubator (e.g., Sanyo, MLR-351H, Osaka, Japan).
17. Autoclaved ddH_2O.
18. Air-permeable tape (e.g., Scotch filter tape, 3 M; St. Paul, MN, USA).

3 Methods

1. Use tweezers to make a small vertical hole at the center of a dissecting plate (*see* **Note 5**).
2. Transfer an intact seedling from MS plate to the dissecting plate near the hole. Using stereomicroscope, transplant the seedling into the hole with the petiole base just touching the agarose surface (*see* Fig. 1a and **Note 6**).
3. Dig out a small piece of wedge-shaped agarose from the periphery of the dissecting plate, and use it to securely wedge the seedling into the hole. Make sure the seedling sits stable (*see* **Note 7**).
4. To detach the first pair of true leaves (i.e., P_{11} and P_{10}), first press the leaf petiole down while gently dragging it left and right to gradually reveal leaf axil and abaxial incision line (the junction between the petiole basal part and the hypocotyl). Use a syringe needle to cut along flanks of the abaxial incision line, and keep dragging outward gently until the leaf is detached (*see* Fig. 1b, c, and **Note 8**). If necessary, cut the vasculature linkage between the detaching leaf and the hypocotyl. Float the detached leaf on the in vitro culture plate with the petiole basal part touching the medium.
5. To dissect the younger leaves (P_9–P_7), use tweezers to gently press the leaf petiole or blade down to reveal the leaf axil. Use a syringe needle to carefully remove two stipules lying on the leaf axil, and then detach the leaf. Collect leaves from one seedling, and transfer to an in vitro culture plate for staining and imaging (*see* Fig. 1d).
6. Transfer one leaf to an imaging plate, and apply one drop of FM4–64 dye solution to the petiole basal part, where AM progenitor cells reside. Check under stereomicroscope to ensure that the basal part is immersed completely in the solution for homogeneous staining (*see* **Note 9**).

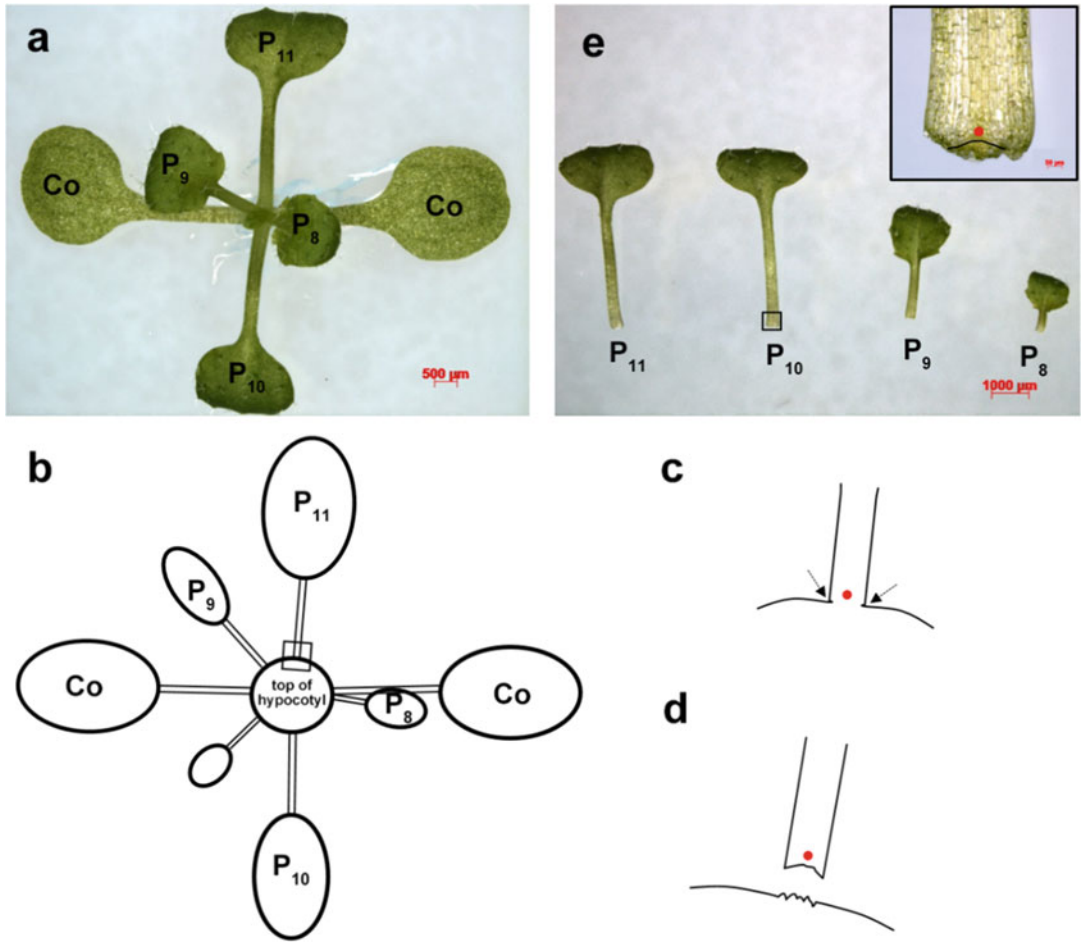

Fig. 1 Detaching leaves for AM imaging. (**a**) A 16-d-old Col-0 seedlings grown under short-day conditions with two cotyledons (Co) and leaves P_{11} to P_7 visible by naked eyes sit in a dissecting plate. (**b**) Diagram of (**a**) to highlight the leaf axils, one of which is enclosed with a black rectangle. (**c**) Schematic of detaching a leaf from the seedling. Arrows indicate flanks of the leaf boundary. The red dot marks AM progenitor cells. (**d**) Schematic of a detached leaf with AM progenitor cells (the red dot) undamaged. (**e**) Leaves (from P_8 to P_{11}) detached from the same seedling floating on an in vitro culture plate. The insert shows corresponding magnified basal part (including the leaf axil) of the P_{10} indicated by a black box. The red dot marks AM progenitor cells, and the black line below indicates the boundary. Bars = 500 μm in (**a**), 1000 μm in (**b**), and 50 μm in the insert of (**b**)

7. After 20 min, transfer the stained leaf to another imaging plate. Under a stereomicroscope, insert the leaf blade into the center of the medium, and adjust the angle of inserted leaf blade until the leaf axil harboring meristem cells is horizontal and facing upward (*see* Fig. 2a, b and **Note 10**).
8. Cover the exposed petiole basal part in the imaging plate with sterile ddH_2O. Remove any air bubbles around the petiole tip by pipetting, and then cover the plate with a lid (*see* **Note 11**).

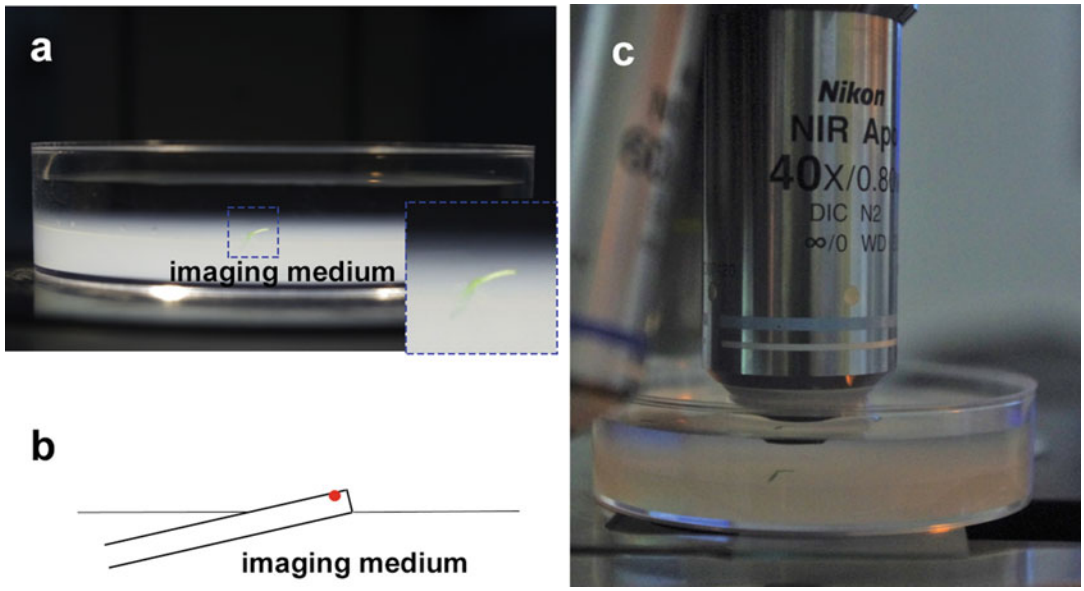

Fig. 2 Preparation of detached leaves for AM imaging. (**a**) A leaf blade inserting in an imaging medium with the leaf axil facing upward, positioned horizontally and covered with water. (**b**) Diagram of (**a**) to highlight the insertion angle. (**c**) View of an imaging plate with a leaf insert in the central part as in (**a**) on a CLSM stage. A 40× water dipping lens is used

9. Position the inserted leaf blade under water dipping lens of CLSM, and roughly aim it to the leaf axil by adjusting the stage. Then, raise the microscope stage until the objective lens immerses into water, but not yet touching the leaf axil (*see* Fig. 2c). Carefully look from all sides to ensure there are no bubbles attaching to the objective lens. Otherwise, lower the stage and redo the immersion.
10. Under epifluorescence illumination, position the leaf axil exactly within the field of the objective lens by further adjusting the stage. Follow the acquisition procedures suggested by the CSLM manufacture to obtain 3D images of the leaf axil (*see* Fig. 3).
11. After imaging, lower the stage, take out the plate, and close its lid to take it back to the hood. After pouring out the water with the help of a pipette, gently drag the leaf out of the hole and transfer it back to a culturing plate. Make sure the petiole basal part touches the medium surface so that the leaf is kept moist and unstressed.
12. Seal the culturing plate with air-permeable tape, and put it in an illumination incubator with short-day conditions.
13. For the following time points (usually at an interval of 24 h), unseal the culturing plate in a sterilized hood and repeat **steps 6–12**. For untreated wild-type plants, it takes 4–5 days to see AM initiation with its own primordia forms.

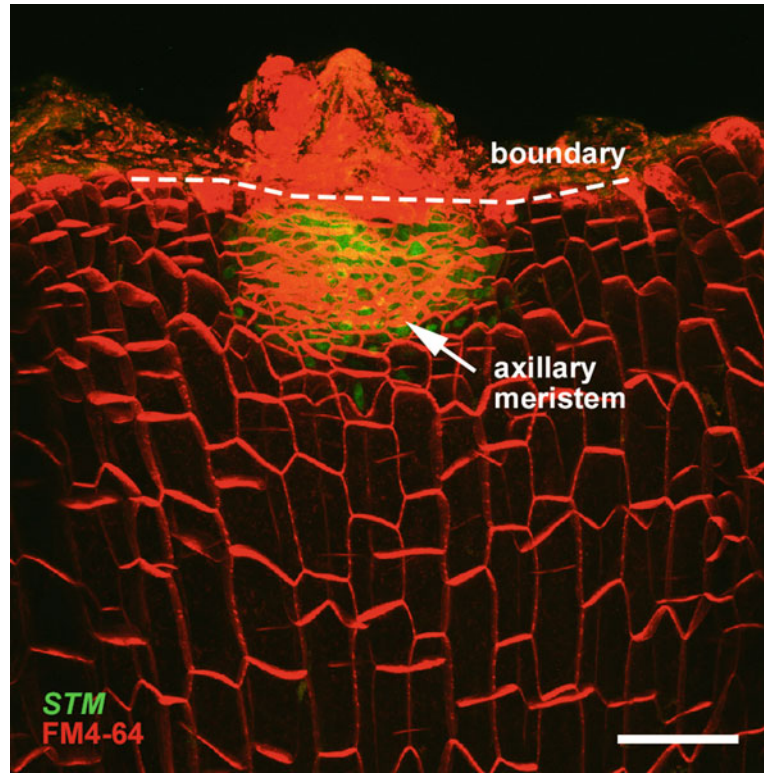

Fig. 3 Maximum intensity projection view of confocal z-stacks of a FM4–64 stained (red) *pSTM::STM-Venus* (green) leaf basal part from a live P_8 2d after in vitro culture. The arrow indicates the AM, and the white dotted line above highlights the boundary. Bar = 50 μm

4 Notes

1. A sterilizer is convenient, but not necessary. Flame sterilization with an alcohol burner also works.
2. Do not over autoclave phytagel.
3. Round edges of glass dishes make it more comfortable to dissect plants inside.
4. Short-day condition is required to avoid precocious AM initiation and axillary bud formation.
5. Stabbing holes in the center of plates provide a convenient angle for dissection.
6. To avoid damaging leaf blade, use tweezers to hold the junction between leaf petioles and the stem to transfer seedlings. Under a stereomicroscope, put the entire roots and hypocotyl into the hole and the stem base above the agarose.

7. It is crucial to ensure the seedling firmly secured in the agarose for dissection. A proper sized piece of wedge-shaped agarose may help.
8. AM progenitor cells are small in size and lie right next to the boundary, i.e., the junction between the petiole base and the stem. Therefore, try to keep the boundary intact to avoid damaging meristematic cells.
9. Once the imaging medium surface is wet, change to a new one and air dry the former one in a hood. The FM4-64 dye will be absorbed into a wet imaging medium surface.
10. It is helpful to make a sloppy path in the medium using tweezers to insert a leaf blade. It is important to insert a leaf blade in the center of a plate to provide enough space for the objective lens.
11. This is to minimize contamination when a plate is taken out of the hood for imaging.

Acknowledgments

We thank Max Bush for schematics and Muhammad Sajjad for proofreading. Research in the authors' laboratory is supported by the National Natural Science Foundation of China grants 31430010, 31861130355, and 31825002, the Key Project of the Frontier Science of the Chinese Academy of Sciences grant ZDBS-LY-SM012, and a Royal Society Newton Advanced Fellowship (NAF/R1/180125).

References

1. Schmitz G, Theres K (2005) Shoot and inflorescence branching. Curr Opin Plant Biol 8:506–511
2. Wang J, Tian C, Zhang C, Shi B, Cao X, Zhang T-Q, Zhao Z, Wang J-W, Jiao Y (2017) Cytokinin signaling activates *WUSCHEL* expression during axillary meristem initiation. Plant Cell 29:1373–1387
3. Shi B, Zhang C, Tian C, Wang J, Wang Q, Xu T, Xu Y, Ohno C, Sablowski R, Heisler MG, Theres K, Wang Y, Jiao Y (2016) Two-step regulation of a meristematic cell population acting in shoot branching in *Arabidopsis*. PLoS Genet 12:e1006168
4. Wang Y, Wang J, Shi B, Yu T, Qi J, Meyerowitz EM, Jiao Y (2014) The stem cell niche in leaf axils is established by auxin and cytokinin in *Arabidopsis*. Plant Cell 26:2055–2067
5. Nimchuk ZL, Perdue TD (2017) Live imaging of shoot meristems on an inverted confocal microscope using an objective lens inverter attachment. Front Plant Sci 8:773
6. Prunet N, Jack TP, Meyerowitz EM (2016) Live confocal imaging of *Arabidopsis* flower buds. Dev Biol 419:114–120
7. Reddy GV, Heisler MG, Ehrhardt DW, Meyerowitz EM (2004) Real-time lineage analysis reveals oriented cell divisions associated with morphogenesis at the shoot apex of *Arabidopsis thaliana*. Development 131:4225–4237