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► **To cite this version:**

Geraldine Brunoud, Carlos Galvan-Ampudia, Teva Vernoux. Methods to Visualize Auxin and Cytokinin Signaling Activity in the Shoot Apical Meristem.. *Methods in Molecular Biology*, 2020, 2094, pp.79-89. 10.1007/978-1-0716-0183-9_9 . hal-03019935

HAL Id: hal-03019935

<https://hal.science/hal-03019935>

Submitted on 24 Nov 2020

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Methods to visualize auxin and cytokinins signalling activity in the shoot apical meristem

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ABSTRACT

Visualizing the distribution of hormone signalling activity such as auxin and cytokinins is of key importance for understanding regulation of plant development and physiology. Live imaging and genetically encoded hormone biosensors and reporters allow monitoring the spatial and temporal distribution of these phytohormones. Here, we describe how to cultivate live shoot apical meristems after dissection for observation under the confocal microscope for up to four days. The shoot apical meristems are maintained on an appropriate medium allowing them to grow and initiate new organs at a frequency similar to plants grown on soil. Meristems expressing hormone biosensors and reporters allows following hormone signalling activity distribution at high spatio-temporal resolution without chemical fixation, an approach that can also be applied to follow the dynamics of expression *in vivo* of any fluorescent marker.

Keys words: shoot apical meristem, phytohormones biosensors and reporters, live imaging, confocal microscopy.

INTRODUCTION

The plant hormones auxin and cytokinins are key regulators of growth and development. Both are small molecules, tryptophan-derived and adenine-derived respectively, which are very difficult to detect *in vivo* at high spatio-temporal resolution. The shoot apical meristem (SAM) is a specialized tissue located at the tip of the growing stem in which these hormones plays a key role. The SAM contains a stem cell niche and produces most of the above ground parts of plants, including stem, leaves, shoots and flowers. In the SAM (as in other parts of the plant), auxin is a central regulator of patterning and organogenesis. It is actively and polarly transported and this transport creates auxin differential distributions or gradients throughout the tissue [1]. This non-homogenous distribution in the tissue is notably essential to organ initiation that is triggered by local accumulation of auxin. Cytokinins have been shown to promote stem cell identity and maintenance in the central zone of the

meristem [2]. They are also regulating the timing of organ initiation at the shoot apical meristem and as a result phyllotaxis robustness [3].

Several sensors and reporters for auxin and cytokinin are currently available. The auxin signalling input can be visualised using the DII-VENUS degradation-based biosensor, or its R2D2 derivative [4, 5], that has been engineered using the degron of an Aux/IAA protein fused to the fast maturing VENUS yellow fluorescent protein and a nuclear localization signal (NLS). Aux/IAA are degraded upon perception of auxin and DII-VENUS fluorescence thus provides information on the combined action of auxin concentration and of auxin perception capacity in plant cells. AUXIN RESPONSE FACTORS (ARFs) and ARABIDOPSIS RESPONSE REGULATORS (ARRs) are transcriptional factors that activate gene expression in response to auxin and cytokinin respectively. Synthetic reporters consisting in multiple ARF or ARR binding sites fused to a minimal promoter and driving a reporter protein allows following the transcriptional activity induced downstream of auxin and cytokinin (signalling output) respectively: auxin-induced transcription can be followed using the DR5 synthetic promoter [6, 7] or the DR5v2 promoter [5] while the TCS and TCSn synthetic promoter allows following cytokinin-induced transcription [8, 9]. These different genetically encoded markers have been largely used to follow auxin distribution and cytokinin signalling at the shoot apical patterning [3, 4, 10-12].

Live imaging microscopy of genetically encoded fluorescent reporters allow following the spatio-temporal dynamics of hormone distribution at the shoot apical meristem during developmental and physiological responses [11-13]. Here we provide a detailed description of how to cultivate live shoot apical meristems after dissection for observation under the confocal microscope for up to four days. We show how using the spatio-temporal distribution of auxin and cytokinin activity can be followed with hormone biosensors and reporters and correlated with key developmental processes such as stem cell maintenance, organogenesis and patterning.

BIOLOGICAL MATERIAL

As introduced above, we use Arabidopsis plants expressing markers constructed using DR5, DR5v2 and TCS promoters to follow hormone-induced transcription. We use here *DR5::3xVENUS-N7* and *DR5v2::ntdTomato* [5, 11]. The two reporters differ not only in the fluorescent protein (FP) driven by the promoter but also in the repetitive DNA sequences bound by ARF (Auxin Responsive Elements –AuxRE-) in the promoter [5]. *DR5v2* has been demonstrated to have a higher sensitivity than *DR5* in certain tissues. For Cytokinin, we use the *TCS::GFP* reporter [8].

To detect rapid changes in auxin concentration and signalling activity, we use the degradation-based biosensors *DII-VENUS* and *R2D2* [4, 5]. *DII-VENUS* is driven by the 35S ubiquitous promoter and *R2D2* consists in a single construct with *DII-VENUS* and *mDII-ntdTomato* driven by two independent *RPS5a* promoters that provide a strong and ubiquitous expression in the SAM. While the DII sequence allows for a rapid degradation by auxin, this degradation is strongly inhibited by the mutation present in the *mDII* sequence. *R2D2* thus provides a ratiometric reading of the auxin-dependent degradation of *DII-VENUS*.

All the Arabidopsis lines used here are in the Col-0 ecotype except *R2D2* that is in the Columbia-Utrecht (Col-utr) ecotype [14].

MATERIALS

1-Half-MS Medium

Prepare medium with:

- Half MS medium without vitamins,
- Sucrose 1%

Adjust pH to 5,8 with 1M KOH

Add Agarose at 0.8% final

Autoclave the medium

Use to pour plates or store at RT once solidified until plates are needed

2-Vitamins stock (1000X)

Myo-inositol	5g
Nicotinic acid	0.05g
Pyrodoxine hydrochlorid	0.05g
Thiamine hydrochloride	0.5g
Glycine	0.1g
Add Water	50ml

Sterilize using 22µm filtration filters, aliquot and store at -20°C and use for no longer than a year.

The aliquots can be frozen and defrozed several times.

3- N6-Benzyladenine (BAP, a cytokinin)

Prepare a 100µM stock solution in water.

To allow for the complete dissolution, add 1% v/v of NaOH 1N.

Sterilize using 22µm filtration filters, store aliquots at -20°C and use for up to six months.

Once defrozed, the aliquots should be discarded after use.

4-Dyes to stain cells contours

-Plasma membrane staining using FM4-64 (N-(3-Triethylammoniumpropyl)-4-(6-(4-(Diethylamino) Phenyl) Hexatrienyl) Pyridinium Dibromide)

Prepare a stock solution at 1mg/ml in sterile water. Store aliquots at -20°C and use for no longer than a year.

Once defrozed, the aliquots should be put back to -20°C only once for maximal staining.

-Cell wall staining using Propidium Iodide (PI, 3,8-Diamino-5-[3-(diethylmethylammonio)propyl]-6-phenylphenanthridinium diiodide)

Use directly solution from Sigma Aldrich available at 1mg/ml (Ref: P4864). Store in fridge at +4°C.

5-Tools for dissection

Forceps: Size N°5 Roth (ref: LH79.1)

Petri dishes: 60X10mm Falcon (ref: 353004)

Porous tape

White and soft facial tissue with high absorption

6-Microscope equipment

-Stereomicroscope

-Confocal microscope upright (Zeiss LSM700) equipped with a long distance objective X40 water immersion (for example we use a Zeiss W "N-Achroplan" 40x/0.75 M27 (ref 420967-9900), water, working distance 2.1 mm, without cover glass).

-Confocal microscope inverted (Zeiss LSM710) equipped with a long distance objective X40 water immersion (for example we use a Zeiss W "N-Achroplan" 40x/1.0 M27 (ref 421462-9900), water, working distance 2.5 mm, without cover glass).

METHODS (Fig1)

1-Preparation of the *in vitro* Apex Culture Medium (ACM) plates

Melt the half-MS medium.

Add vitamins (final concentration 1X) and BAP (final concentration 200nM) under a laminar hood, sterile conditions (see note1). Pour the medium in Petri dishes.

Use directly or store at +4°C for no longer than a month.

2-Plant growth conditions

The following growth conditions are required:

- Short day conditions: 8 hours light and 16 hours night, 21°C, hygrometry 55-60%.
- Long day conditions: 16 hours light at 21°C and 8 hours night at 18°C, hygrometry 55-60%

To obtain healthy plant and facilitate dissection, plants are germinated in short day condition and grown in these conditions for 4 weeks. Plants are transferred for two weeks in long day conditions to induce flowering and development of the inflorescence (optimal conditions, Fig1A). Water the plants at least two hours before dissection. It is possible to dissect meristems from plants grown in long days only or secondary and axillary meristems but the smaller size of the stem makes the dissection more difficult.

3-Dissection (Fig1B, C, D, E)

Dissect from 6 to 24h before the first observation (see, note 2).

First by naked eye, remove as much as possible of the older flowers with the forceps (Fig1B). Then using the stereomicroscope, there are two alternative ways to continue with the dissection depending on the length of the inflorescence stem. Meristems with stems from 5 to 30 mm long can be dissected directly on the plant (Fig1C). Inflorescences longer than 30 mm can be cut and dissect on medium plate, by cutting at 1cm for the top and transferring the segment of stem onto the medium plate (Fig1D). Then remove old primordia one by one using forceps, try to cut the petioles more closely to stem as possible. The meristem appears at the centre as a green dark tissue in comparison with the primordia (Fig2), (see note 3).

A last methods for very long stem and old plant: cut the stem at their based and placed horizontally on a piece of craft paper and dissected by rolling the stem with the fingers (Fig1E) and remove all lateral organs progressively using forceps (see note 4).

After dissection, transfer the meristems to clean medium plates (see note 5 and note 6) that will be used for microscope acquisition (Fig1F), taking care of aligning the stem with the gravity axis. Close the plates with porous tape and place the dishes back in the culture chamber or in a growth incubator with similar growth conditions.

5-Before microscopy

Use a stereo microscope to check the dissected SAM as the sample might require additional dissection. Again, it is also important to check that the meristem is vertically aligned (Fig1F and Fig2).

Submerge the meristem with sterile water for 8 minutes by filling the dish (see note 7).

6-Staining of the cell contours (optional)

Stain meristems with PI (at 100 μ M final concentration in water) for 5 minutes (see note 8). In order to reduce staining volumes, we place a sterile 1000 μ L tips (cutted about 2 cm from the top of the tip) around the dissected sample and add sufficient PI solution (about 150 μ l) to fully cover the meristems (Fig 1G). After incubation, rinse twice with sterile water (Fig1H), (see note 9).

Alternatively SAMs can be stained with FM4-64. Add FM4-64 (at 300 μ M final concentration in water) for 10 minutes by applying 10 μ l (one drop) on top of the dissected meristem. The drop of FM4-64 solution should stay stably on the SAM for optimal staining. Rinse briefly twice with sterile water.

7-Acquisition

With an upright microscope (Fig1I), place the imaging dish onto the stage, then lower the microscope lens until it touch the surface of the water (see note 10), be careful to avoid air bubbles under the lens.

With an inverted microscope (Fig1J), apply a drop of sterile water on the lens, hold the imaging dish upside down on the stage then raise the microscope lens until a water column is formed between the lens and medium(see note 10 and note 11).

Using transmitted light, position the sample within field of the objective, then focus on the top of the meristem using the Z control. It is also possible to use the epifluorescence illumination to find the meristem, however this might lead to fluorescence bleaching of the sample and should be avoided if not absolutely necessary.

Adjust the confocal settings (laser power, gain, offset) accordingly to the signal. To avoid signal saturation, use the range indicator palette, and set the gain until there is no or few red points in the image. It is recommended to use a minimal laser power to avoid photo-bleaching. It is also possible to open the pinhole up to 1.5 or 2. We use the following settings: for VENUS (DR5, DII and R2D2) laser 514nm (5% power) and emission range: 520-560nm, for ntdTomato (R2D2) laser 561nm (5% power) and emission range: 580nm-620nm and GFP (TCS) laser 488 (5% power) and emission range 467-537nm. For PI and FM4-64 we used the 488nm laser at 5% power and adjust the emission range from 646 to 660 nm to avoid auto fluorescence interference [15] (see note 12).

8-After imaging

When doing a time course experiment (see note 13 and note 14), it is important to remove the excess of water from the sample. Under the stereomicroscope, the meristem has to appear shiny and without any sign of water on it or on the medium before putting it back to grow. To remove all the

water after imaging, use a clean absorbent facial tissue to absorb any drop on the medium and let the plate dry for 4 to 6 minutes before closing it. When necessary, remove older organs after image acquisition not before to avoid stressing the meristem (see note 15).

9-Analysis/post processing

For image processing and signal quantification, ImageJ or Fiji (<http://imagej.net/Welcome> or <https://imagej.net/Fiji>) offer multiple tools. It is out of our scope to describe all of them here and we are just giving here a few hints on how to analyse the data. Maximal intensity Z-projections help to visualise the distribution of the different markers. Figure 3. Show projections obtained using Fiji: in A; nuclear DR5 signal (LUT fire) and cell wall stained by PI (in yellow), in B; nuclear DII (LUT blue orange icb) and auto fluorescence (in red), in C; reticulum endoplasmic TCS signal (LUT Green Fire Blue) and auto fluorescence (in red), in D; R2D2 line with the DII signal (LUT Rainbow) and the constitutive expression (in green).

Further quantification and analysis of meristems can require tissue segmentation and 3D reconstruction. For this MorphoGraphX MGX (<http://www.mpipz.mpg.de/MorphoGraphX/> P.), [16] or MARS-ALT (<http://openalea.gforge.inria.fr/doc/vplants/vtissue/doc/build/html/user/index.html> [17] can be used. We refer the reader to the corresponding website and publications for use of these approaches

NOTES

1. Wait for medium temperature to reduce to 50-55°C to avoid denaturation of vitamins and BAP.
2. Let the meristem recover from the dissection. Mechanical or wounding stresses are to be avoided as they can affect the fluorescence distribution of the reporters. DII is particularly sensitive and dynamics, the DII signal completely disappears within minutes after the dissection but reappears progressively.
3. The dissection should be as rapid as possible to limit desiccation of the meristem.
4. The rolling method induces more mechanical stresses and a faster desiccation of the sample, but can be very useful when the plants have grown more rapidly than expected.
5. Let the plate at room temperature at least 30 minutes before using to avoid cold stress to the meristem.
6. Dissect a double number of meristems than the number you expect to image and analyse because some of them will not survive over a long time course.
7. It is crucial to immerse the sample in water few minutes before imaging to avoid sample movement during acquisition due to water absorption and swelling of the gel.
8. PI staining reveal damage, because when the tissue is damaged the PI will penetrate inside and the signal will be strong and/or in the nucleus rather than well localized at the cell wall.
9. The cut 1000 µL tips can then be reused. They need then to be kept in Ethanol 70% to avoid contamination.

10. Use a water immersion objective to avoid desiccation, if dry objective is used the imaging time should be less than one minute.
11. During long imaging time regularly check the water column and add water if necessary.
12. When imaging meristems with different fluorophores, we recommend sequential (multi-track) acquisition to avoid cross-channel signal contamination.
13. Bacteria or fungus contamination will kill the meristem. To avoid this use a new bottle of sterile water at each imaging time. The objective has also to be cleaned before imaging with 70% ethanol.
14. For long time courses (more than 2 days), the ACM medium has to be change (every other day) to avoid contamination but also be certain that the BAP concentration is stable.
15. A way to know the meristem is still alive and in good condition is that you should detect new organ initiation at around one new organ per 12 hours.

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FIGURE LEGENDS

Fig1: Illustration of the experimental procedure. A: optimal size of the plant for the dissection. B, C, D, E: several steps of the dissection procedure as described in the text. F: Meristems on an ACM plate at the end of the dissection. G: Set-up for PI staining. H: Wash after staining. I, J: Set-up for acquisition using upright (I) and inverted (J) microscope. K, L: Visualization of fluorescence in a meristem on a Zeiss LSM710 inverted confocal microscope and Zen associated software.

Fig2: Visualization under the stereomicroscope of a dissected Shoot Apical Meristem on the ACM medium. Note the darker colour of the meristem at the center. Scale Bar: 50µm

Fig3: Maximum projections performed using Fiji.

A: *DR5::3VENUS-N7* expression in LUT fire, cell wall stained by PI in yellow. B: *DII-VENUS* in LUT blue orange icb, auto fluorescence in red. C: *TCS::erGFP* in LUT Green Fire Blue, auto fluorescence in red. D: *R2D2; DII* in LUT Rainbow, constitutive expression in green. Scale bar 50µm