This is a post-peer-review, pre-copyedit version of an article published in Methods in Molecular Biology. The final authenticated version is available online at: https://doi.org/10.1007/978-1-62703-221-6_12

Small molecule screens to study lateral root development

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Abstract

Development of the root system is essential for proper plant growth and development. Extension of the root system is achieved by the continuous establishment of new meristems in existing parental root tissues, which leads to the development of lateral roots. This process of lateral root organogenesis consists of different developmental stages, which are all controlled by the plant hormone auxin. In this chapter, we describe a screening method in *Arabidopsis thaliana* to identify small synthetic molecules that interfere with the process of lateral root development during specific developmental stages.

1. Introduction

Roots are of high importance for proper growth and development of plants. They do not only provide water and nutrients to the plant but are also required for stable anchorage in the soil. In addition, roots are the first line of defense against biotic and abiotic stresses. A typical property associated with the root system is its extreme flexibility and plasticity. Because of the sessile nature of plants, root plasticity is essential to retrieve new sources of nutrients and water. This is achieved by the ability of roots to continuously develop new branches from existing root tissue, which eventually leads to an extended network of root branches.

In dicotyledons, a single primary root develops from the radicle during the process of germination. During root growth and development, root branching occurs by the addition of lateral roots that originate from xylem-pole pericycle cells of the primary root. Lateral root development can be divided in distinct developmental stages (1). At the primary root tip, more specifically in the region of the basal meristem, pre-initiation events specify founder pericycle cells from which a lateral root will develop. Functional studies have shown that the control of lateral root founder cell identity is mediated by the GATA23 transcription factor (2). Higher up in the root, cell cycle is activated in the pericycle founder cells, and the first cell divisions occur that lead to the formation of a lateral root primordium (Vanneste 2005, De Smet 2008 erbij?). Subsequently, the activated pericycle cells proliferate through the overlying cell layers i.e. endodermis, cortex and epidermis, and eventually emerges from the primary root (3). This active process is mediated by the LAX3 efflux carrier (4).

The plant hormone auxin is a main regulator of lateral root development during every developmental stage (1, 2, 4). As such, the application of auxin, auxin-analogues and auxin antagonists makes it extremely difficult to pinpoint and study a particular lateral root developmental stage (5). The availability of small synthetic molecules that inhibit and/or activate a specific developmental step would aid tremendously in this respect. However, in order to obtain these specific molecules, several thousands of compounds need to be screened. Inevitably, this requires a high-throughput screening assay that highlights a lateral root developmental stage of interest. Here, we describe a miniaturized expression-based screening approach in transgenic *Arabidopsis thaliana* seedlings, based on the Lateral Root Inducible System (LRIS, Himanen 2004, Vanneste 2005), that is compatible with high-throughput screening technology. Dependent on the marker gene, the method allows identifying small molecules that interfere with a specific process during lateral root development.

2. Materials

2.1. Plant material

1. Transgenic *Arabidopsis thaliana* seeds that are homozygous for a marker construct (**Notes 1** and **2**)

2.2. Seed sterilization

1. Sterile distilled water.
2. 70% (v/v) ethanol solution.
3. 5% (v/v) NaOCl / 0.05% (v/v) Tween 20 solution.

2.3. Primary and confirmation screening

1. 96-well filter plates (MSBVS1210, Millipore) with plastic lids.
2. Porous tape for air-permeable sealing.
3. Sterile distilled water.
4. Half-strength Murashige and Skoog (MS) growth medium consisting of 0.215 g/L MS salts supplemented with 10 g/L sucrose, 0.1 g/L myo-inositol and 0.5 g/L 2-(N-morpholino)ethanesulfonic acid (MES) monohydrate in distilled water. Adjust the final pH to 5.7 by adding 1 M KOH. Autoclave the medium at 1 bar overpressure for 20 min. Growth medium can be stored at 4 °C.
5. 50 mM (14.57 mg/mL) N-(1-naphthyl)phthalamic acid (NPA) in 100% dimethyl sulfoxide (DMSO) (**Note 3**).
6. 50 mM (9.31 mg/mL) 1-naphthaleneacetic acid (NAA) in 100% DMSO (**Note 3**).
7. Collection of small molecules, dissolved in 100% DMSO at a concentration of 5 mM, pre-plated in 96-well plates (Corning Life Sciences) and stored at -20 °C (**Notes 4** and **5**).
8. Continuous light growth chamber (110 µE/m^2s photosynthetically active radiation, supplied by cool-white fluorescent tungsten tubes, Osram) at 22 °C.
9. Orbital shaker (IKA KS 260 basic).
10. Vacuum manifold (MSVMHTS00, Millipore).
11. Vacuum/pressure pump (WP6122050, Millipore).

2.4. Analysis

1. 90% (v/v) acetone solution.
2. 200 mM 5-bromo-4-chloro-3-indolyl β -D-glucopyranosid, sodium salt (X-Glc) in 100% dimethyl formamide (DMF) (**Note 3**).
3. Triton X-100.
4. 500 mM ethylenediaminetetraacetic acid (EDTA) in distilled water. Adjust the final pH to 8 by adding 10 N NaOH. EDTA solution can be stored at room temperature.
5. Phosphate buffer: mix 615 mL of a 500 mM Na_2HPO_4 solution and 385 mL of a 500 mM NaH_2PO_4 solution. Phosphate buffer can be stored at room temperature.
6. 100 mM potassium ferricyanide ($K_3Fe(CN)_6$) in distilled water. Ferricyanide solution can be stored at -20 °C.
7. 100 mM potassium ferrocyanide ($K_4Fe(CN)_6$) in distilled water. Ferricyanide solution can be stored at -20 °C.
8. Dissecting microscope.

3. Methods

3.1. Seed sterilization

1. Add dry transgenic *Arabidopsis thaliana* seeds that contain the reporter construct of interest in a sterile 2 mL microcentrifuge tube (**Note 6**).
2. Add 70% ethanol for 2 min.
3. Remove the 70% ethanol solution and add 5% NaOCl / 0.05% Tween 20 solution for 15 min.
4. Remove the 5% NaOCl / 0.05% Tween 20 solution and wash five times with sterile distilled water. Leave water in the tubes after the last wash and put at 4 °C for at least 2 days.

3.2. Preparation of assay plates

1. Prepare the growth medium by adding NPA to half-strength MS medium to a final concentration of 10 µM (**Note 7**).
2. Add 150 µL of the growth medium to 96-well filter plates by using a multichannel pipette.
3. Add between 3 and 10 sterilized seeds per well by using a 200 µL pipette with tips of which the top is cut to increase the opening size (**Note 8**).
4. Seal the plates with plastic lids and air-permeable tape.
5. Put the plates in the continuous light growth chamber under continuous shaking (150 rpm).
6. Incubate for 5 days.

3.3. Distribution of compounds

1. Remove the NPA-containing growth medium by vacuum filtration (**Note 9**).
2. Add 147 µL of half-strength MS medium to the plates by using a multichannel pipette.
3. Add 1.5 µL of the compound screening collection (5 mM, 100% DMSO) to the plates (columns 2 to 11) by using a multichannel pipette (**Note 10**).
4. Add 1.5 µL of a 100% DMSO solution to columns 1 and 12.
5. Seal the plates with plastic lids and air-permeable tape.
6. Put the plates in the continuous light growth chamber under continuous shaking (150 rpm).
7. Incubate for 2 h (**Note 11**).
8. Prepare a 1 mM NAA solution (2% DMSO) by diluting a 50 mM NAA solution (100% DMSO) with sterile distilled water.
9. Prepare a 1 mM NPA solution (2% DMSO) by diluting a 50 mM NPA solution (100% DMSO) with sterile distilled water.
10. Add 1.5 µL of the 1 mM NAA solution (2% DMSO) to columns 1 to 11, add 1.5 µL of the 1 mM NPA solution (2% DMSO) to column 12 (**Notes 12** and **13**).
11. Seal the plates with plastic lids and air-permeable tape.

12. Put the plates in the continuous light growth chamber under continuous shaking (150 rpm).
13. Incubate for 24 h (**Note 14**).

3.4. Analysis

1. Remove the growth medium by vacuum filtration.
2. Add 150 µL of a 90% aceton solution to the plates by using a multichannel pipette.
3. Incubate for 30 min at 4 °C (**Note 15**).
4. Remove the 90% aceton solution by vacuum filtration.
5. Wash 3 times with phosphate buffer.
6. Prepare the GUS staining solution by adding 1.5 mL of a 200 mM X-Glc solution in 100% DMF, 1.5 mL Triton X-100, 0.6 mL of a 500 mM EDTA solution, 1.5 mL of a 100 mM potassium ferricyanide solution and 1.5 mL of a 100 mM potassium ferrocyanide to a final volume of 300 mL phosphate buffer.
7. Remove the phosphate buffer by vacuum filtration.
8. Add 150 µL of the GUS staining solution to the plates by using a multichannel pipette. (**Note 16**).
9. Incubate for several hours at 37 °C (**Note 17**).
10. Remove the GUS staining solution by vacuum filtration.
11. Wash 3 times with phosphate buffer (**Note 18**).
12. Analyze the effect of the compounds on the expression pattern of the marker gene by using a dissecting microscope (**Note 19**).
13. Identify wells with compounds that interfere with the expression pattern of the marker gene under study (**Note 20**).

4. Notes

1. Typically, transgenic *Arabidopsis thaliana* lines contain a construct consisting of the promoter of a marker gene (gene-of-interest) fused to a reporter gene (β-glucuronidase, GUS) to visualize expression of the marker gene.
2. Dependent on the lateral root developmental stage of interest, different marker genes can be considered; for example *GATA23* for lateral root founder cell specification in the pre-initiation stage (2), *CYCB1;1* for the lateral root initiation stage (4), *IAA3* for lateral root protrusion through the endodermis (5) and *LAX3* for lateral root protrusion through the cortex (4).
3. DMSO- and DMF- dissolved chemical reagents should be freshly prepared from dry powder.
4. DMSO is a hygroscopic solvent. The absorption of water leads to compound degradation, reduced solubility and changes in compound concentration and as such

affects the overall quality of the chemical library. This can be circumvented by storing the DMSO-dissolved compound collections in low-humidity environments such as under nitrogen in enclosed containers. In addition, because collections are stored in frozen condition (-20 °C), solutions need to be thawed to access them and refrozen for further storage. Freeze/thaw cycles should be kept to a minimum because they have dramatic effects on compound integrity because of precipitation and/or sample degradation (6).

5. Several companies supply pre-plated diverse sets of DMSO-dissolved small molecules that can be used for screening purposes. These companies include ChemBridge Corporation, Enamine, Life Chemicals, Maybridge, Asinex,…
6. Screening of 20 96-well plates requires about 500 µL of dry seeds.
7. The addition of NPA inhibits auxin transport in the developing primary root during the germination process. Consequently, all seedlings have a primary root without any additional lateral roots. This allows synchronization of the lateral root development process over all seedlings, which results in a more robust screening assay.
8. The number of seeds per well does not need to be exactly the same in each well because the analysis is qualitative (assessment of changes in expression pattern) rather than quantitative.
9. After each vacuum filtration step, tap the bottom of the plates with paper tissue to remove remaining drops.
10. Screening collections are mostly delivered in 96-well plates with columns 1 and 12 left empty, which allows adding controls in each assay plate.
11. A pre-incubation step will allow the synthetic molecules to penetrate the root cells previous to the activation of lateral root development by NAA treatment.
12. This approach aims at the identification of small molecules that inhibit lateral root development at the developmental stage reported by the marker gene. Removing the step of NAA addition to the compound-treated wells will lead to the identification of small molecules that activate lateral root development.
13. Column 1 will serve as a positive control (enhanced lateral root development), column 12 will serve as a negative control (absence of lateral root development).
14. Because pre-initiation events occur as early as 2 h after NAA treatment and cell cycle activation (lateral root initiation) occurs at 6 h after NAA treatment, shorter incubation times can be considered to identify compounds that interfere with these early lateral root developmental stages.
15. Longer incubation times should be avoided as this will affect the filters at the bottom of the plate, which could eventually result in leakage of the plates in subsequent steps.
16. To analyze 20 plates, at least 300 mL of GUS staining solution is required.
17. The expression pattern of both positive and negative controls should be checked regularly during the incubation. When the difference between positive and negative

controls is easily scoreable, GUS staining can be stopped. Total incubation time is dependent on the marker gene and the *Arabidopsis thaliana* GUS line. Typically, incubation times range between 2 and 24 h.

18. At this stage, plates can be stored at 4 °C for several weeks prior to analysis of the expression pattern.
19. On average, scoring of an entire 96-well plate takes about 15 min.
20. Compounds that are identified as hits should be retested by using the same protocol to confirm their effect.

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