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/10.1007/978-1-62703-592-7_1

Fully-automated compound screening in *Arabidopsis thaliana* seedlings

Dominique Audenaert[1,2], Long Nguyen[3], Bert De Rybel[4], Tom Beeckman[1,2]

1 Department of Plant Systems Biology, VIB, Technologiepark 927, B-9052 Gent, Belgium

2 Department of Plant Biotechnology and Bioinformatics, Ghent University, Technologiepark 927, B-9052 Gent, Belgium

3 VIB Compound Screening Facility (VIB-CSF), Technologiepark 927, B-9052 Gent, Belgium

4 Current address: Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

Abstract

High-throughput small molecule screenings in model plants are of great value to identify compounds that interfere with plant developmental processes. In academic research, the plant *Arabidopsis thaliana* is the most commonly used model organism for this purpose. However, compared to plant cellular systems, *Arabidopsis thaliana* plants are less amenable to develop high-throughput screening assays. In this chapter, we describe a screening procedure that is compatible with liquid handling systems and increases the throughput of compound screenings in *Arabidopsis thaliana* seedlings.

1. Introduction

In chemical biology, low molecular mass molecules are applied as conditional tools to reveal the underlying mechanisms that control growth and development. Chemical biology approaches have been well established to study human biology and disease mechanisms (1, 2). Also in plant sciences, several studies have shown that chemical biology represents a powerful tool to study plant developmental processes (3, 4).

The identification of small molecules that interfere with a biological process of interest requires screening of a large number of compounds in an assay that is compatible with high-throughput screening. During the past two decades, affordable collections of small

molecules have become commercially available. This has allowed academic researchers to perform chemical screenings on large sets of compounds. But although pre-plated compound collections are readily available, academic researchers still face the major challenge of developing a proper and robust screening assay. In plants, cellular systems such as *Arabidopsis thaliana* protoplasts or tobacco Bright Yellow-2 cells are relatively easy to adapt to a screening format due to its compatibility with high-throughput screening robotics (5). However, because cellular systems lack a proper physiological context, they are less applicable to study developmental processes. The identification of compounds that affect specific developmental and physiological processes requires the availability of a screening assay in a plant model system. *Arabidopsis thaliana* is a model organism that is frequently used to study plant growth and development because of its available genetics and genomics tools. In addition, *Arabidopsis* can germinate and grow until the young seedling stage in 96-well plates and consequently *Arabidopsis* assays are amenable to high-throughput screening purposes. Indeed, this has been demonstrated by several studies in which a large number of compounds were screened in *Arabidopsis* in 96-well plate format (6, 7).

Although adding compounds to the assay plates can relatively easy be automated using a liquid handling platform, a major limitation of *Arabidopsis* assays is the high degree of manual handling for distributing the seeds and performing the read-out. Consequently, the throughput of screenings in *Arabidopsis* is significantly inferior compared to cell-based assays. In this chapter, we describe a method to fully automate compound screenings in *Arabidopsis thaliana* seedlings. We have developed a procedure to add seeds to 96-well plates via liquid handling robotics and to perform a plate reader-based read-out. This method allows greatly improves the throughput of screenings using *Arabidopsis thaliana*.

2. Materials

2.1. Plant material

1. Transgenic *Arabidopsis thaliana* seeds

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. Seed distribution and germination

1. 0.1% (w/v) sterile agar solution.
2. White 96-well filter plates (MSBVN1B50, Millipore) with plastic lids.
3. Half-strength Murashige and Skoog (MS) liquid 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.

4. Liquid handling robotic system (**Note 1**).
5. Porous tape for air-permeable sealing (1530-0, 3M Micropore™).
6. *Arabidopsis thaliana* growth chamber with controlled light conditions and temperature.
7. Orbital shaker (IKA KS 260 basic).

2.3. Compound distribution and incubation

1. 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 (**Note 2**).
2. Liquid handling robotic system (**Note 3**).
3. Vacuum manifold (MSVMHTS00, Millipore) and vacuum/pressure pump (WP6122050, Millipore) (**Note 4**).
4. Half-strength liquid MS growth medium.
5. Porous tape for air-permeable sealing (1530-0, 3M Micropore™).
6. *Arabidopsis thaliana* growth chamber with controlled light conditions and temperature.
7. Orbital shaker (IKA KS 260 basic).

2.4. Read-out

1. ONE-Glo Luciferase Assay System (E6130, Promega) (**Note 5**).
2. Liquid handling robotic system (**Note 6**).
3. Plate reader (**Note 7**).

3. Methods

3.1. General considerations

For automated plate reader-based read-outs, the method requires the use of a transgenic *Arabidopsis thaliana* line that contains a construct consisting of the promoter of a marker gene (gene-of-interest) fused to the gene encoding firefly luciferase or green fluorescent protein (GFP) to visualize expression of the marker gene. Using GFP does not require the addition of a substrate solution, which makes the screening faster and less expensive. Furthermore, unlike luminescence-based read-outs, which are endpoint measurements, GFP can be used for multiple time-point measurements. However, a major disadvantage of using GFP as a reporter is that autofluorescent compounds might interfere with the assay read-out, requiring an auto-fluorescence screening of the chemical library purchased to ignore auto-fluorescent compounds.

Typically, the marker gene needs to meet several criteria to be applicable in this method.

1. Up- or down-regulation of the marker gene is associated with the biological process under study. This requires thorough characterization of a marker gene that is as specific as possible for the process. Identification of a suitable marker gene can be achieved by previously performed microarray experiments.
2. Changes in the expression level of the marker gene need to be as large as possible to allow changes being detectable via a plate reader.
3. Preferably, expression of the marker gene is increased upon changes of the biological process. Using marker genes that show a reduced expression level may lead to false positive hits due to toxicity. For any setup, hit compounds should be further characterized using complementary assays.

3.2. Seed sterilization

1. Add dry transgenic *Arabidopsis thaliana* seeds in a sterile 15 mL Falcon tube (**Note 8**).
2. Add 10 mL of a 70% ethanol solution for 2 min.
3. Remove the 70% ethanol solution and add 10 mL of a 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, seeds are vernalised at 4 °C for at least 24 h.

3.3. Seed distribution and germination

1. Add half-strength MS growth medium to a container that is compatible with the liquid handling platform (**Note 1**).
2. Add 135 µL of the growth medium to the white 96-well filter plates.
3. Prepare the seed distribution solution by adding 0.3 mL or 0.6 mL of the sterilized seeds to 10 mL of a 0.1% agar solution (**Fig. 1** and **Note 9**).
4. Add the seed distribution solution to a container that is compatible with the liquid handling platform (**Note 1**).
5. Add 15 µL of the seed distribution solution to the white 96-well filter plates.
6. Seal the plates with plastic lids and air-permeable tape.
7. Put the plates in the continuous light growth chamber at 21 °C under continuous shaking at 150 rpm (**Note 10**).
8. Incubate for 5 days (**Note 11**).

3.4. Compound distribution and incubation

1. Remove the growth medium by vacuum filtration (**Note 12**).
2. Remove the air-permeable tape and plastic lids from the plates.
3. Add 148.5 µL of growth medium to the white 96-well filter plates (**Note 1** and **Note 13**).

4. Add 1.5 µL of a negative control to column 1 and 1.5 µL of a positive control to column 12 (**Note 14**).
5. Add 1.5 µL of the compound screening collection (5 mM, 100% DMSO) to columns 2 to 11 (**Note 3** and **Note 15**).
6. Seal the plates with plastic lids and air-permeable tape.
7. Put the plates in the continuous light growth chamber at 21 °C under continuous shaking at 150 rpm (**Note 10**).
8. Incubate for 24 h (**Note 16**).

3.5. Analysis

1. For luminescence-based read-outs, prepare the ONE-Glo luciferase substrate solution according to the manufacturer's protocol (**Note 17**).
2. Add 100 µL of the prepared luciferase substrate solution and incubate for about 5 min.
3. Load the plates into the plate reader and perform the read-out (**Note 18**).
4. Identify wells with compounds that interfere with the expression level of the marker gene under study.

4. Notes

1. For distribution of seeds and growth medium, our laboratory has access to a Beckman Coulter Biomek 2000 Laboratory Automation Workstation. To allow subsequent seed germination under sterile conditions, the platform is placed in a custom-made laminar flow. The platform has the capacity to process eight plates in one run. The addition of the growth medium and seeds to these plates takes about 25 min in total. Per screening batch, we perform three runs, which adds up to a total of 24 plates.
2. 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,…
3. For compound distribution, our laboratory has access to a Tecan Freedom EVO200 platform with 96-Multi Channel Arm option, a Robotic Manipulator Arm and an integrated carousel with barcode scanner. This setup allows full walk-away automation during compound distribution, which leads to a significant increase in throughput.
4. The vacuum manifold and vacuum/pressure pump are integrated in the Tecan Freedom EVO200 platform.
5. Only required for luminescence-based read-outs.
6. The Tecan Freedom EVO200 platform is used to add the ONE-Glo reagent to the assay plates.

7. At the laboratory, we have a FLUOstar OPTIMA (BMG Labtech) available for fluorescence intensity measurements and a LUMIstar OPTIMA (BMG Labtech) for luminescence measurements.
8. Automated distribution of *Arabidopsis* seeds requires about 1 mL of dry seeds for 24 96-well plates.
9. Sterilized seeds should be aspirated slowly from the bottom of the Falcon tube with a 2 mL sterile plastic pipette. The amount of seeds to aspirate and dispense in the 0.1% agar solution is dependent on the desired final number of seeds per well as described in Fig. 1. A total of 10 mL of the seed distribution solution suffices to perform one run (eight plates) on the Biomek 2000 platform. The 0.1% agar solution prevents the seeds from settling down in the container during dispensing in the 96-well plates.
10. We have experienced optimal germination under these conditions. However, light, temperature and shaking conditions can be adjusted dependent on the assay.
11. Germination and initial growth occur in the absence of chemicals to prevent excessive toxicity during the early developmental stages. However, the time of growth in the absence of compounds can be decreased or increased dependent on the developmental process under study. For example, early root growth studies may require shorter times whereas leaf development would require longer times.
12. Integration of the vacuum manifold and vacuum/pressure pump in the Freedom EVO200 platform allows removal of the growth medium of the entire screening batch (i.e. 24 plates) in a fully automated fashion.
13. At this stage, the growth medium might contain additional components (e.g. plant hormones) to induce or inhibit a developmental process.
14. As a negative control, we add 1.5 µL of a 100% DMSO solution. The positive control is dependent on the biological process under study. For example, auxin is used as a positive control for the process of lateral root development. Control compounds are applied with the Biomek 2000 platform.
15. This will yield a final compound concentration of 50 µM in the plates. The screening collection is applied with the Freedom EVO200 platform.
16. The usual compound incubation time that we apply is 24 hours. However, dependent on the biological process under study, incubation times can be adjusted.
17. This step can be omitted for fluorescence-based read-outs.
18. Integrating the plate reader with a plate stacker would allow to fully automate the read-out. Importantly, with luminescence-based read-outs, the batch size to process should not be too large to avoid the luminescent signal to decrease over time. Therefore, after the substrate is added with the liquid handling robotics, the read-out should occur within 1 h. Taken that measuring an entire 96-well plate takes about 3 min, the total batch for luminescence measurements should not exceed 20 plates.

References

1. Collins, I., Workman, P. (2006) New approaches to molecular cancer therapeutics. *Nat Chem Biol* **2**, 689-700.
2. Gangadhar, N.M., Stockwell, B.R. (2007) Chemical genetic approaches to probing cell death. *Curr Opin Chem Biol* **11**, 83-87.
3. Robert, S., Chary, S.N., Drakakaki, G., Li, S., Yang, Z., Raikhel, N.V., Hicks, G.R. (2008) Endosidin1 defines a compartment involved in endocytosis of the brassinosteroid receptor BRI1 and the auxin transporters PIN2 and AUX1. *Proc Natl Acad Sci USA* **105**, 8464-8469.
4. Kim, T.H., Hauser, F., Ha, T., Xue, S., Böhmer, M., Nishimura, N., Munemasa, S., Hubbard, K., Peine, N., Lee, B.H., Lee, S., Robert, N., Parker, J.E., Schroeder, J.I. (2011) Chemical genetics reveals negative regulation of abscisic acid signaling by a plant immune response pathway. *Curr Biol* **21**, 990-997.
5. De Sutter, V., Vanderhaeghen, R., Tilleman, S., Lammertyn, F., Vanhoutte, I., Karimi, M., Inzé, D., Goossens, A., Hilson, P. (2005) Exploration of jasmonate signalling via automated and standardized transient expression assays in tobacco cells. *Plant J* **44**, 1065-1076.
6. Armstrong, J.I., Yuan, S., Dale, J.M., Tanner, V.N., Theologis, A. (2004) Identification of inhibitors of auxin transcriptional activation by means of chemical genetics in Arabidopsis. *Proc Natl Acad Sci USA* **101**, 14978-14983.
7. De Rybel, B., Audenaert, D., Vert, G., Rozhon, W., Mayerhofer, J., Peelman, F., Coutuer, S., Denayer, T., Jansen, L., Nguyen, L., Vanhoutte, I., Beemster, G.T., Vleminckx, K., Jonak, C., Chory, J., Inzé, D., Russinova, E., Beeckman, T. (2009) Chemical inhibition of a subset of Arabidopsis thaliana GSK3-like kinases activates brassinosteroid signaling. *Chem Biol* **16**, 594-604.

Figure

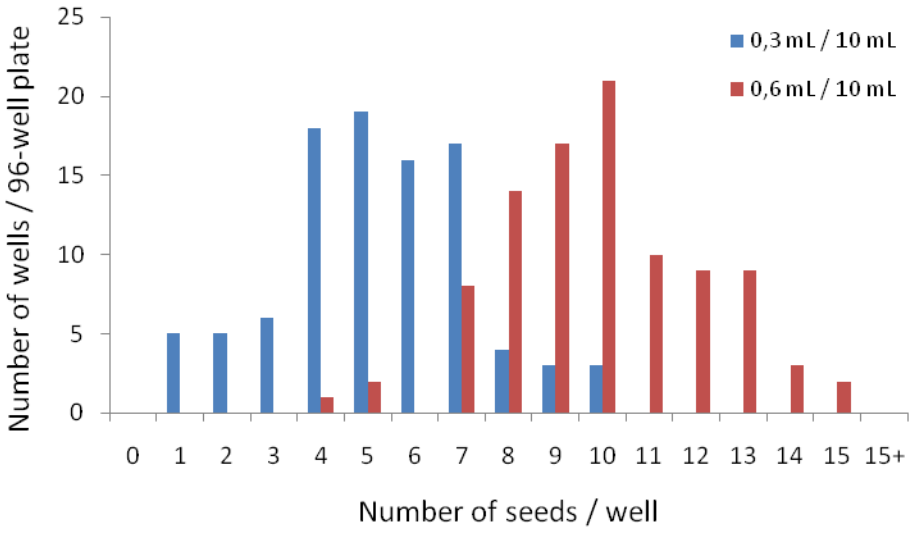

Fig. 1. Number of wells that contain the indicated number of seeds after dispensing 15 µL of the seed distribution solution. With a seed concentration of 0.3 mL / 10 mL, the final seed number is between 3 and 7 seeds per well for 75% of the wells. With a seed concentration of 0.6 mL / 10 mL, the final seed number is between 7 and 11 seeds per well for 75% of the wells.