

A Chemical Genetic Screening Procedure for *Arabidopsis thaliana* Seedlings

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[Abstract] Unbiased screening approaches are powerful tools enabling identification of novel players in biological processes. Chemical genetic screening refers to the technique of using a reporter response, such as expression of luciferase driven by a promoter of interest, to discover small molecules that affect a given process when applied to plants. These chemicals then act as tools for identification of regulatory components that could not otherwise be detected by forward genetic screens due to gene family redundancy or mutant lethality.

This protocol describes a chemical genetic screen using *Arabidopsis thaliana* seedlings, which has led to recognition of novel players in the plant general stress response.

Materials and Reagents

1. Reporter line seeds (this protocol was developed using luciferase under control of a minimal promoter containing four copies of the rapid stress response element - *4xRSRE:LUCIFERASE*)
2. Murashige and Skoog basal medium (Sigma-Aldrich, catalog number: M0404)
3. Phytoagar (PlantMedia, catalog number: 40100072-2)
4. Sterile disposable reagent reservoir (Corning, catalog number: 07-200-128)
5. Bleach (Clorox concentrated, 8.25% sodium hypochlorite)
6. Hydrochloric acid (HCl)
7. Chemical library (source may vary)
8. Potassium luciferin (Gold Biotechnology, model: LUCK-1G)
9. ¼ strength MS media (see Recipes)
10. 1 mM luciferin (see Recipes)

Equipment

1. Micropore surgical tape (3M, model: 1530-0)
2. 1.5 ml tubes (SealRite, catalog number: 1615-5500)
3. 96 well plates with lid: Flat bottom, sterile but not tissue culture treated (SARSTEDT AG, catalog number: 82.1571.001)

4. Filter paper (here used Whatman 1440 125, pore size does not matter)
5. Toothpicks
6. Laminar flow hood or biosafety cabinet (here used SterilGARD ii, the Baker Company)
7. Chemical fume hood (here used St. Charles)
8. Vacuum chamber
9. Multichannel pipette (2-20 and 20-200 µl) (Rainin)
10. Temperature-controlled growth cabinet (here used Conviron, model: GR48)
11. Charge-Couple Device camera (CCD camera; Andor DU434-BV CCD)
12. Dehesh lab Perl scripts
(<http://www.plb.ucdavis.edu/labs/dehesh/dehesh-lab-code.html>)

Software

1. ANDOR Solis analysis software (Andor technology, v15,
<http://www.andor.com/scientific-software>)
2. ImageJ image analysis software (<http://imagej.nih.gov/ij/>)

Procedure

A. Preparation

1. To synchronize germination and improve developmental consistency among seedlings, pre-select the larger and more uniformly sized seeds from selected *Arabidopsis thaliana* (*Arabidopsis*) reporter line by pouring seeds several times along a sheet of paper. The smallest seeds tend to stick to paper via electrostatic forces and can easily be discarded.
Note: Using seeds harvested at the same time, from plants grown under the same conditions, will also improve seedling uniformity.
2. Autoclave filter paper, toothpicks, and filter tips for 200 µl pipet. Surface-sterilize size-selected seeds:
 - a. Aliquot approximately 50-100 µl size-selected seeds in 1.5 ml tube with chlorine-resistant label.
 - b. In chemical fume hood, place open 1.5 ml tubes in rack in vacuum chamber with beaker containing ~100 ml bleach.
 - c. Add 3 ml HCl to beaker and close vacuum chamber.
 - d. Apply vacuum for 5-10 sec to seal chamber.
 - e. After 2.5-3 h, open chamber (still in fume hood) and close lid of 1.5 ml tubes.
3. Prepare solid ¼ strength MS media according to the recipe below.
Note: This protocol is for seeds grown on solid media, but similar screens have been performed on plants grown in liquid media.

B. Plating seeds

1. "Pour" MS media into 96-well plates. Inside sterile hood, pour molten media into sterile reagent reservoir. Set multichannel pipet at desired volume + 10% (for 100 μ l, set at 110). Draw media up from reservoir and eject into 96-well plate 1 row at a time.

Notes:

- a. *Bubbles in the wells make plating seeds difficult. To minimize the incidence of bubbles and mis-filled wells, (a) touch the pipet tips to the corner between bottom and side of well before ejecting and (b) do not eject the media fully, only to the first stop of the multichannel pipet. This results in some media remaining in pipet tips, but greatly reduces bubbles.*
 - b. *If you do not work quickly enough, media may solidify in the reservoir or pipet tips. To avoid this we recommend starting with >65 °C media. If it becomes a problem, simply eject and replace tips or reservoir.*
2. Pour sterilized seeds onto filter paper. Using moistened tip of sterile toothpick, pick up and place one seed per well of the 96-well plate.
Note: This allows a second opportunity for seed size selection. We observe significant differences between responses of border wells, at the edge of the plate, and internal wells. You should test for this difference and if necessary do not apply treatments or measure border wells. If border wells will not be measured then place any remaining smaller seeds in these, using uniformly larger seeds for internal wells.
 3. Tape edges of 96-well plates with micropore surgical tape to maintain sterility and humidity.

C. Seedling growth

1. Stratify seeds for 3-5 days in the dark at 4 °C.
2. Grow seedlings under typical *Arabidopsis* plate growth conditions (22-24 °C under 16 h light (60-80 μ E) /8 h dark).
3. Six days after placing under light, seedlings should have expanded cotyledons and two visible true leaves, ready for treatment with luciferin (Figure 2A). To reduce background it is best to proceed with luciferin treatment 18-24 h before imaging.

Note: We apply luciferin via spray, with detergent (see Recipes). To reduce false positives/negatives one must ensure uniform substrate availability by saturating all wells. In screening we achieved this uniformity by spraying twice from approximately 6 inches above the plate for each of left and right sides of the plate, followed by three sprays from approximately 9 inches above the plate tracking from left to right.

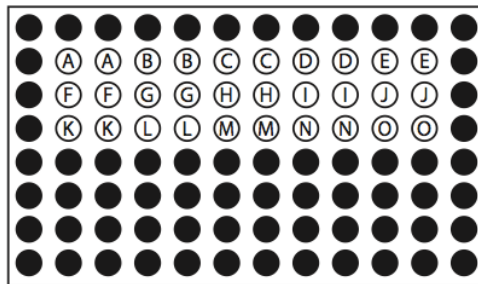
D. Imaging

1. At seven days post-stratification, move plates from growth chamber to the lab bench several hours prior to imaging.

Note: In the case of 4xRSRE:LUCIFERASE-expressing seedlings, plates were moved

at 8 am, four hours before imaging at noon. This was done to minimize modulation of physiological responses, and by extension luciferase activity, associated with changing plant environment. The time interval between moving and imaging may vary depending on the nature of the experiment, but beginning imaging at the same time throughout screen is advisable due to possible interaction with circadian-regulated processes.

Chemical template plate: 12*4 = 48 μ L per well



Treatment Plates: 10 μ L per well

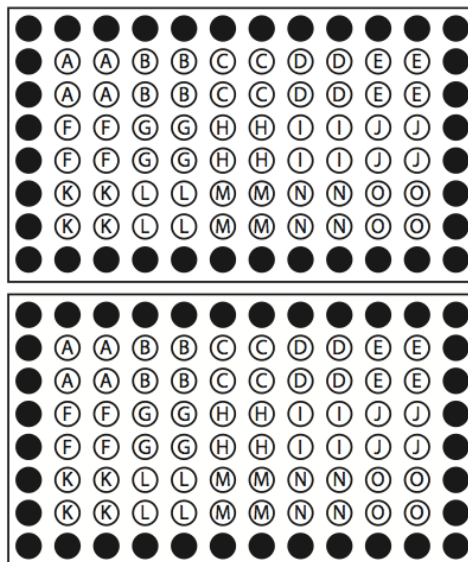


Figure 1. Organization of chemical template plate and corresponding treatment plates. In this example row 2 of the chemical template plate is used to treat rows 2 and 3 of both sample plates, row 3 of chemical template plate is used to treat rows 4 and 5 of both sample plates, and row 4 of the chemical template plate is used to treat rows 6 and 7 of both sample plates.

2. Prepare chemicals 1: Dilution. It will likely be necessary to adjust the concentration of the chemical library by diluting in water or other appropriate solvent before treatment, based on the output observed. In our experiments we observed optimal working concentrations between 20 and 40 μ M-few chemicals had any visible effect below 10 μ M. Chemicals may be applied as a 10 μ l drop at working concentration to treatment

wells; dilute each chemical to produce a total volume sufficient for 12 μ l per well to account for pipetting errors.

Note: Some chemical solvents, such as dimethyl sulfoxide (DMSO), may affect the output signal. Prior to a large-scale screen, one should check for solvent effects at desired dilution level. We recommend a “diluted solvent” negative control, in addition to a no treatment control and where possible a positive control, on each plate.

3. Prepare chemicals 2: Template plate. Prepare a chemical template plate on ice, with working concentration of chemicals in the same arrangement as they will be applied to treatment plate. We recommend applying each chemical to at least 8 replicate wells. This should yield at least 6 suitable seedlings to measure response. If replicate wells are placed in columns, one row on the chemical template plate may apply to multiple rows on the treatment plate (Figure 1).

Notes:

- a. *If border wells will not be assayed this must be reflected in the chemical template plate.*
- b. *The number of seedlings devoted to each chemical treatment should be determined by variability in the system of interest and practical constraints, i.e. the number of chemicals to be tested.*

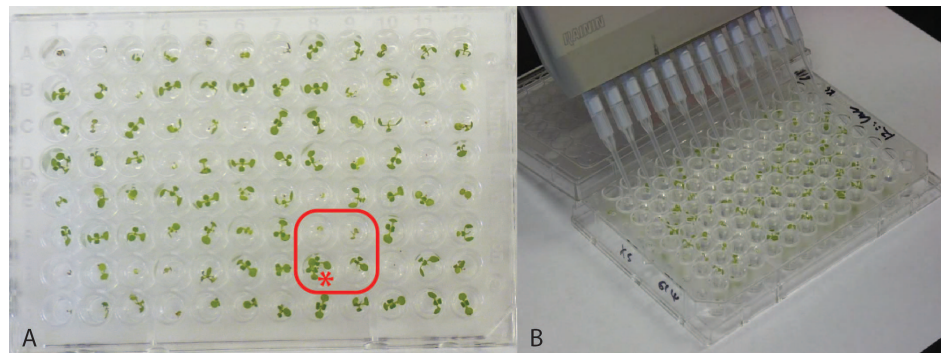


Figure 2. Representative 96-well plates. (A) 7-day old seedlings. Note the set of four wells boxed in red, reflecting two of the potential issues in plating. This set of wells shows poor germination resulting in plants too small for reliable analysis, and two seedlings erroneously germinated in one well (marked with *), eliminating them for analysis. Panel (B) shows multichannel pipet chemical application.

4. Treat seedlings by transferring chemicals from template to treatment plate using multichannel pipette. It is important to not disturb the seedlings-if seedlings are wounded by a pipet tip it may change how chemicals are perceived and thus their response. We recommend touching the pipet tips to well sides away from seedlings, as close to agar as possible in order to minimize splashing or chemical adhesion to the side of the well (Figure 2B). Once all chemicals have been applied gently rock the plate for ~5 sec to ensure even chemical distribution in wells.

Note: In this screening technique, it is not defined how the plant takes up the chemical. The chemical droplet may be taken up from the surface of the agar through the hypocotyl/cotyledon/leaves, in which case the working concentration is perceived. Alternatively, the chemical may be taken up through the roots after chemical diffusion into media. In this case, the media itself further dilutes the chemical and the perceived concentration may be as little as 1/10 the applied working concentration (10 μ l chemical applied diluted into 100 μ l media).

5. Imaging conditions will depend on reporter used, but we recommend imaging for up to triple the time of typical response, to allow for delayed response as chemical diffuses through media, etc. We used a 20 h time course of five minute exposures of a CCD camera within a light-tight box, controlled by the ANDOR Solis v15 image analysis software (<http://www.andor.com/scientific-software>).

E. Analysis/re-screening

1. Depending on the intensity and uniformity of response, an initial qualitative overview may be sufficient for first-round screening, to be followed by a quantitative analysis (Figure 3).
 - a. For rapid quantification of combined information on what fraction of each seedling's total area is luminescent and how bright that luminescence is, we used custom Perl scripts to interpret ImageJ analysis of images exported from ANDOR Solis v. 15. The commented scripts are available at <http://www-plb.ucdavis.edu/labs/dehesh/>. Briefly, this technique requires users to place a grid of round Regions Of Interest (ROI) in the ImageJ software using the plugin Microarray Profiler. ImageJ quantifies the "white" value for each pixel in these ROI, which can then be normalized to plant area. This technique allows for rapid screening of a large number of wells, but it does not distinguish between the pattern of luciferase activity (*i.e.* number of pixels with some non-zero "white" value) and the intensity of emitted light (*i.e.* magnitude of "white" value).
 - b. For more defined quantification, small square ROI can be placed one-by-one on individual cotyledons using the ANDOR software. This eliminates the effect of area of luminescence while achieving greater precision in intensity. We found 4pixel-by-4pixel ROI to be an optimal size for seven-day-old *Arabidopsis* cotyledons. Commented Perl scripts for this analysis are also available at <http://www-plb.ucdavis.edu/labs/dehesh/>.

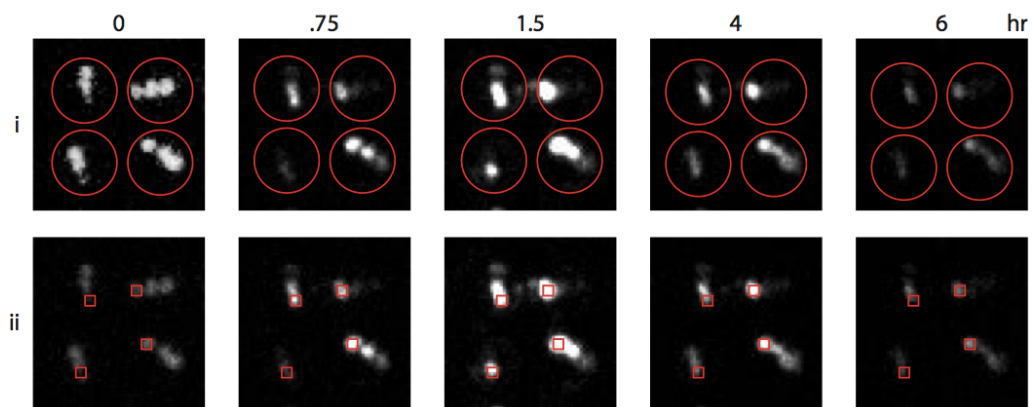


Figure 3. Model data demonstrating two analysis methods. Shown are the same set of four wounded *Arabidopsis* seedlings at selected time points up to 6 h. In method (i), a grid of ROI (red circles) fits over each whole seedling. Luciferase activity is quantified as a fraction of theoretical maximum (picture at timepoint 0 with contrast enhanced). In method (ii), square ROI are placed on individual cotyledons, providing greater precision.

2. Once putative chemical “hits” have been identified via first-round screening, they must be confirmed in a second and possibly third round. Second round screening should proceed similarly to first round, with the exception that we recommend at least doubling or preferably tripling the number of wells devoted to each chemical, followed by the more carefully defined quantification of response as described in step E1b.

Note: In the first round of screening, we identified approximately 15% of screened chemicals as putatively affecting 4x RSRE: LUCIFERASE activity. Second round screening confirmed ~10% of these putative chemicals. These numbers should be expected to change depending on the output, sensitivity of reporter, and chemical library selected.

3. Confirmed chemical hits may lead to multiple avenues of experimentation, including concentration curves to determine active concentration, developmental time course of chemical effect, analysis of selected chemicals similar in structure or known function, and/or analysis of chemical effect on plant beyond screen output.

Notes

General screening notes

1. The screen output described here is luminescence from luciferase driven by a minimal promoter containing four copies of the RSRE, but the screening technique may be applied to any reporter system, or visible effects on *Arabidopsis* seedlings. Simply substitute any pretreatment for luciferin application, necessary imaging equipment for the CCD camera/ANDOR software, and appropriate image analyses.

2. This screen is designed to observe the immediate effect of chemical treatment, avoiding any secondary effects produced during prolonged exposure. However, if desired, the chemical treatment could be added to molten or solid media before placing seeds in the individual wells, or at any desired stage of *Arabidopsis* growth.
3. If adding chemicals early, as well as during pretreatment with luciferin, take care to minimize contamination risks (*i.e.* use sterile water, keep containers closed as much as possible and if necessary work within sterile laminar flow hood). Contamination may affect the screening output, either directly or through effects on plant fitness.

Recipes

1. $\frac{1}{4}$ strength MS media

Dissolve 1.11 g MS salts in 1 L milli-Q pure H₂O, and adjust pH to 5.7 with NaOH

Measure out 8 g phytoagar into bottle, and pour in pH-adjusted MS solution

Autoclave, liquid cycle, 30 min

Note: Although $\frac{1}{4}$ strength MS media was used here, $\frac{1}{2}$ MS or other plant growth media may work as well or better, and should be tested prior to screening.

2. 1 mM luciferin

Prepare 10x (31.824 mg/10 ml) or 100x (318.42 mg/10 ml) stock solution of potassium luciferin in milli-Q H₂O

Dilute down to 1 mM in sterile H₂O, add Tween-20 to final concentration of 0.01%

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