Chapter 2

Assessing Gravitropic Responses in *Arabidopsis*

Richard Barker, Benjamin Cox, Logan Silber, Arash Sangari, Amir Assadi, and Patrick Masson

Abstract

Arabidopsis thaliana was the first higher organism to have its genome sequenced and is now widely regarded as the model dicot. Like all plants, *Arabidopsis* develops distinct growth patterns in response to different environmental stimuli. This can be seen in the gravitropic response of roots. Methods to investigate this particular tropism are presented here. First, we describe a high-throughput time-lapse photographic analysis of root growth and curvature response to gravistimulation allowing the quantification of gravitropic kinetics and growth rate at high temporal resolution. Second, we present a protocol that allows a quantitative evaluation of gravitropic sensitivity using a homemade 2D clinostat. Together, these approaches allow an initial comparative analysis of the key phenomena associated with root gravitropism between different genotypes and/or accessions.

Key words *Arabidopsis thaliana*, Gravitropism, Gravity perception, Time-lapse photography, Clinostat

1 Introduction

Gravity has been a constant factor in the evolution of life forms on earth, and organisms have evolved strategies to utilize this directional cue to their advantage. For instance, land plants have acquired the ability to utilize the gravity vector as a guide for organ growth (gravitropism), typically directing the roots downward into the soil for plant anchorage and water and nutrients uptake, and also guiding the shoots upward to access light for photosynthesis, exchange gases, and contribute to reproduction. This implies that each organ possess gravity-sensing machinery that allow them to identify changes in their orientation within the gravity field and respond by redirecting growth. In roots, gravitropism prevails amongst directional growth responses to the environment; competing tropic pathways triggered by light, touch, water gradients, salt, and/or oxygen, modulate the effectiveness of gravitropism when their contribution is needed most [1–3].

Paula Duque (ed.), *Environmental Responses in Plants: Methods and Protocols*, Methods in Molecular Biology, vol. 1398,
DOI 10.1007/978-1-4939-3356-3_2,

Many years of experimentation have contributed to elucidating some of the mechanisms that mediate gravity sensing and signal transduction, including investigations on the role of amyloplast sedimentation within specialized gravity sensing cells (the statocytes) in the gravitropic responses of roots and shoots, studies of the molecular mechanisms that allow organs to redistribute the plant hormone auxin upon gravistimulation, identification and characterization of molecules that contribute to auxin transport and response (reviewed in Refs. [4–6]).

In this manuscript, we describe methods that are often used in the laboratory to evaluate: (1) the sensitivity of plant organs to gravistimulation; and (2) the kinetics of curvature response to gravistimulation. Indeed, these two procedures allow a careful evaluation of two critical components of gravitropism: (1) Gravisensing and (2) Properties of curvature response.

For many years, investigators have attempted to evaluate the ability of plant organs to respond to different doses of gravistimulation with distinct levels of curvature. One method has relied on rotating the plants to a defined angle from the gravity vector, and maintaining this orientation for distinct, short periods of time. At the end of each time period, the plants are transferred to a clinostat, which is a slowly rotating device that randomizes the orientation of the plant within the gravity field. Plants are allowed to grow on the rotating clinostat for a few hours, permitting the development of a tip curvature whose angle is a direct function of the gravistimulation dose (time × g) provided before clinorotation.

Using this experimental setup, Larsen [7] demonstrated that a logarithmic (L) model provides an adequate fit to the data, linking angle of curvature to the logarithm of the dose of gravistimulation (defined as time of gravistimulation at 1 g under this experimental setup). He then proposed that the extrapolation of this model to the *X* axis (curvature angle = 0) is a good estimate of the gravisensitivity of the investigated organ, and called it the Presentation Time (minimal gravistimulation time needed to trigger a productive gravity signal transduction pathway). This parameter has often been used as estimate of organ gravisensitivity [reviewed in 8 and 9].

It should however be cautioned that a hyperbolic (H) model can be fitted equally well or better to the same experimental data linking the curvature response to the time of gravistimulation before clinorotation, as previously discussed in Perbal et al. [8]. In fact, in most cases, the H model will be more strongly correlated to the data than the L model [8]. Therefore, Dr. Perbal and his collaborators proposed to use this H model to fit the data, and suggested that the slope of the H curve at the origin is, in fact, a better estimate of gravisensitivity than the presentation time. Hence, in this case, one assumes that even infinitesimal times of gravistimulation are sufficient to trigger a minute response. Because recent investigations have shown that these models fit quite nicely

the data with similar, though not identical correlation values, most researchers use both the H and L models to estimate gravisensitivity. In the first section of this paper, we detail the method we use to estimate root gravisensitivity of different genotypes or accessions, using either *Arabidopsis thaliana* or *Brachypodium distachyon* as models.

Different genotypes (mutants or accessions) will develop distinct kinetics of gravitropic curvature upon gravistimulation, and a careful evaluation of the characteristics of these curvature-response curves provides important information on their distinct properties, such as: the latency period, the speed of curvature during the log phase; the ability to, or time needed by the organ to, resume growth at the gravity set point angle; the final angle of growth from the gravity vector after reorientation; the existence of discontinuities in the shape of the curve suggesting the involvement of distinct mechanisms over time [reviewed in Refs. 4 and 10]. In the second section of this paper, we describe a high-throughput method that allows us to investigate the kinetics of root gravitropism at high temporal resolution for many genotypes in parallel, using a robotic assembly with a mounted camera similar to that described in Brooks et al. and Wells et al. [11] and [12].

Together, these two protocols are being used quite effectively to implement genome-wide association studies (GWAS) of gravitropism using *Brachypodium distachyon* as a model.

2 Materials

2.1 General Materials

1. Square, non-gridded 100 mm petri dishes.
2. Agar Type E (Sigma-Aldrich, St Louis, MO).
3. ½ Strength Murashige and Skoog nutrient salts (Sigma-Aldrich).
4. 20 % Bleach (10 ml bleach, 40 ml H_2O, and 50 μl SDS) or 90 % (v/v) ethanol.
5. A standard controlled growth environment with the following abiotic conditions: Fluorescent light—225 μmol/m^2; Temperature—21–22 °C; Humidity—65–90 %; Long-day cycles: 16 h light and 8 h of darkness.

2.2 2D Clinostat Materials

1. Flatbed scanner (Epson Perfection V33).
2. Clinostat petri-dish holder: details available for download http://masson.genetics.wisc.edu/wp-content/uploads/sites/32/2014/07/Clinostat-Description1.pdf (Fig. 1).
3. Wheaton Roller Culture Apparatus.
4. Timer.

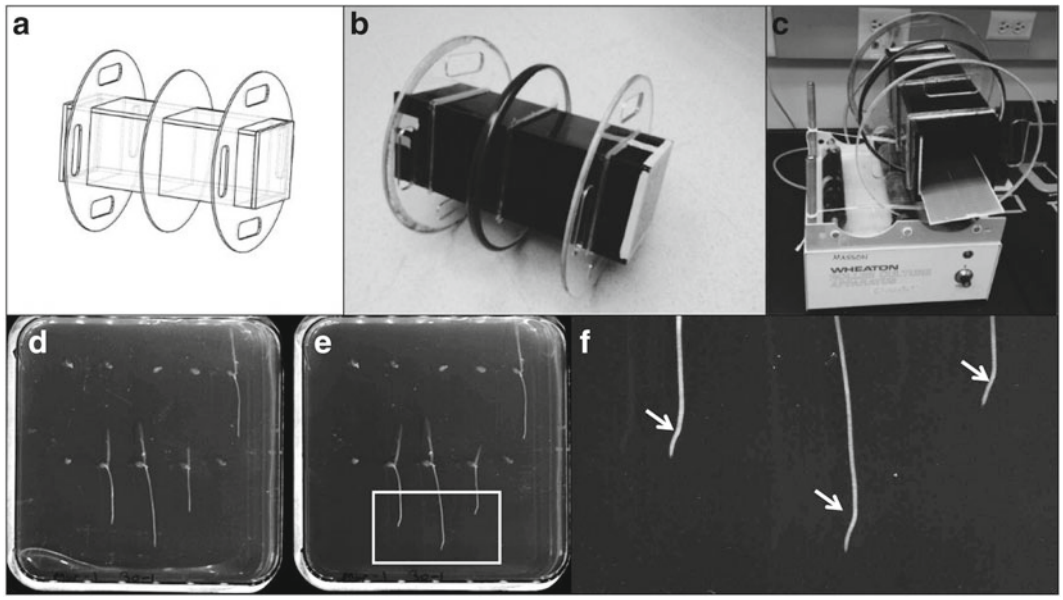

Fig. 1 (**a**) The AutoCAD design of the 2D clinostat petri dish holder. (**b**) This design allows 18 petri dishes to be rotated simultaneously. (**c**) Wires were used to ensure clinostat stayed on the center of the rotator in order to reduce friction with the roller. (**d**–**e**) Example image scanned before and after clino-rotation. (**f**) Schematic diagram highlighting the curvature induce by the gravitational observed 4 h after a clino-rotation

2.3 Time-Lapse Photography Robotic Assembly Materials

1. Camera (we use a Canon EOS rebel XSi with a Canon macro lens EF-s 60 mm 1:2:8 USM).
2. Two STAC6 applied motion stepper drivers and two NEMA 34 stepper motors that move the camera along an 80/20® Inc. aluminum stock track.
3. Custom robot and camera control software to determine the speed, timing, and distance at which the camera moves [13].
4. Four 120 V 2 ft lamps each containing T5 high emission growth light fixtures and 4 fluorescent bulbs illuminate the seedlings.
5. ROSCO E-colour+ #90 dark yellow green filter.
6. 64-bit Microsoft Windows 7 operating system running on a Dell PC with a 2.5 GHz Phenom™ II X4 905e Processor with 4GB of RAM to perform image analysis.
7. Adobe Bridge Camera Raw digital darkroom and Adobe Photoshop Creative Cloud.
8. ARRT software to measure images [14].

3 Methods

3.1 Measure Sensitivity to Gravity Using a 2D Clinostat Protocol

1. Pour molten 1 % agar containing ½ LS salts into petri plates in a laminar flow cabinet and allow it to harden.
2. Submerge *Arabidopsis* seeds for 15 min in 20 % bleach to surface sterilize them and then wash four times with distilled water to remove bleach solution (*see* **Note 1**).
3. Sow seeds horizontally on the agarose gel and seal plates (*see* **Note 2**).
4. Store the plates at 4 °C for 3 days in darkness provided by wrapping the plates in aluminum foil.
5. Place plates vertically in the controlled environment (CE) for 4 days.
6. Remove plates from the CE and scan the plates to create 600 dpi JPEG images (*see* **Note 3**).
7. Leave the plates vertical for 1 h in darkness.
8. Gravitationally stimulate the seedlings by rotating the plates 90°, and incubate in darkness for 10, 20, or 30 min (*see* **Note 4**).
9. Rotate the plates in the clinostat at 1 revolution per minute (RPM) for 4 h.
10. Remove from the clinostat then scan again.
11. Align the pre- and post-rotation scans next to each other using Adobe Photoshop or similar software.
12. Measure the angle of initial root tip curvature developed after transfer to the clinostat (*see* **Note 5** and Fig. 1).
13. For each gravistimulation time, quantify and plot the average angle of curvature with associated variance, fit the corresponding data with L and H model curves as defined in Perbal et al. [8], and use these models to calculate the presentation time (L model) and the sensitivity score (H model) (*see* **Note 6**).

3.2 Automated Time-Lapse Photography of Root Growth and Curvature Response to Gravistimulation

1. Submerge *Arabidopsis* seeds for 15 min in 20 % bleach to sterilize them and then wash four times with distilled water to remove bleach solution (*see* **Note 1**).
2. Sow seeds horizontally on 1 % agar containing ½ LS salts by placing the seeds on the surface of the agarose gel using a pipette or an autoclaved toothpick (*see* **Note 2**).
3. Store the plates at 4 °C for 3 days in darkness obtained by wrapping the plates in aluminum foil.
4. Place plates vertically in the controlled environment (CE) for 4 days.
5. Remove plates from CE and insert them into a petri dish holder that is 1 ft away from the lights, providing approximately

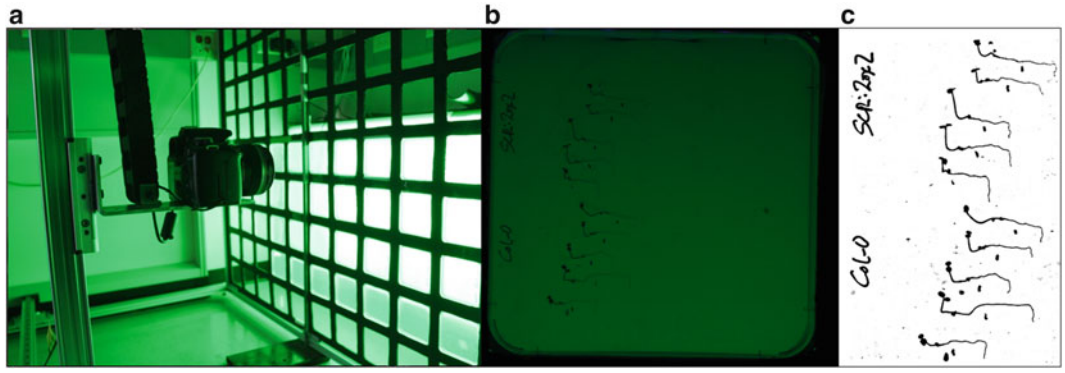

Fig. 2 (**a**) Semi-automated robotic photography system automates the time lapse photography of up to 40 petri dishes. (**b**) The plant growth parameters can be measured in the original photos by hand using ImageJ, Adobe Photoshop or other standard image analysis software. (**c**) Custom image enhancement scripts can digitize the images making it easier to analyze the whole time-lapse series using specific root tip tracking software

70 μmol m^{-2} s^{-1} of light. The room was kept between 22 and 24 °C and had a humidity between 50 and 60 % in front of the camera (*see* **Note 7**).

6. Rotate plates 90° and set the camera to photograph every 30 min for 12 h (*see* **Note 8** and Fig. 2).
7. Optimize root contrast against agar using image enhancement software (*see* **Note 9** and Fig. 2).
8. Measure images using a semi-automated image analysis software package such as ARTT by Russino et al. [14] (*see* **Notes 10** and **11**).
9. Plot data using MS Excel or alternative software package (*see* **Note 12**).

4 Notes

1. Seed can also be sterilized by rinsing with 90 % ethanol three times, then allowed to air-dry onto sterile filter paper.
2. When sealing the plates use medical micro-pore tape before removing the plates from the laminar-flow cabinet to prevent microbial contamination. When working with *Arabidopsis*, it is important to carefully choose the taping material to prevent problems associated with ethylene accumulation [15]. If working with *Brachypodium* or rice submerge the end of the kernel where the radical emerges within the agar.
3. When scanning plates ensure they are parallel to the edge of the scanner. Set up three groups of plates: one for a 10 min stimulus, one for a 20 min stimulus, and another for a 30 min stimulus.

4. When providing the gravitational stimuli the plates should be inside the clinostat chamber as this will provide a dark environment reducing phototropic morphological changes, and it will also minimize the mechanical stimulation. The chamber should not be rotating during this period, of course.
5. Align the two images taken either before or after the clinorotation next to each other. First measure the root tip angle before the plants were gravistimulated. Then find the same location on post clinorotation and measure the new orientation that the root takes.
6. In the L-model ($\alpha = \lambda + \mu.\log10(d)$) where α corresponds to the gravitropic response and d is the dose of the stimulus, we plot the curvature at each time point on a log scale using Microsoft Excel. We then perform a linear regression to calculate the value where the logarithmic line of best fit intercepts the *X* axis. In the Hyperbolic model ($\alpha = a*d/(b+d)$), d is the time (min), α is the gravitropic response (angle of curvature), while a and b are parameters that we want to estimate. Therefore we are estimating two parameters; S = a/b and R^2 = 1- SSres/SStot. We use Wolframs Mathematica to analyze the clinostat results using the H-model running this code:

   In Wolframs Mathematica this will deliver the key parameters of the hyperbolic model

```
a, b, S and R^2wherer = sqrt(R^2)
d = {10, 20, 30};
alpha = {9.73, 13.933, 24.10};
SStot = Total[(Mean[alpha] - alpha)^2];
abOpt = NMinimize[Total[((a d)/(b + d) - alpha)^2], {a, b}];
d1 = 0;
d2 = 40;
alphamin = 0;
alphamax = 40;
Show[Plot[(a d)/(b + d) /. abOpt[[2]], {d, d1, d2},
PlotRange -> {{d1, d2}, {alphamin, alphamax}}],
ListPlot[Table[{d[[i]], alpha[[i]]}, {i, 1, 3}],
PlotMarkers -> {Automatic, 10}, PlotStyle -> Red,
PlotRange -> {{d1, d2}, {alphamin, alphamax}}]]
SSres = abOpt[[1]];
Print["SSres: ", SSres]
Print["SStot: ", SStot]
Slopes = Table[D[(a d[[i]])/(b + d[[i]]) /. abOpt[[2]]],
{i, 1, 3}]
Print["Slope of Fitted Curve at given times: ", Slopes]
Print["a: ", a /. abOpt[[2]]]
Print["b: ", b /. abOpt[[2]]]
Print["S: ", a/b /. abOpt[[2]]]
Print["R^2: ", 1 - SSres/SStot]
```

7. It is important to ensure the plates are filmed in an environment with appropriate temperature and humidity. These can be monitored using a Hobo® data logger inserted into the petri dish holder. In order to remove the negative phototropic effect of the backlight on root growth we use a ROSCO E-colour+ #90 dark yellow green filter to remove blue and red light. Alternatively an infrared camera and far red LED illumination can be used [12].
8. To ensure each plant is the same age when it is photographed each plate should be rotated 90° no more than 1 min before the first photo in the time series is taken.
9. Enhancing images using Adobe Photoshop: A custom image enhancement script was recorded using Adobe Photoshop to prepare the time series for quantification by ARTT [14]. The precise parameters will depend on the image acquisition parameters; these can be easily adjusted using either Adobe Bridge Camera Raw tool or automated batch processing scripts within Adobe Photoshop. Use a naming system that allows you to track the original image source so you can check the times between and add a 3-digit serial number so that the images stay in sequence. Select a new folder location to save them; this may take a long time depending on the number of images you are working with. Below is an example of a script designed to make roots slighty larger and bolder to aid with subsequnt analysis. The parameters to adjust using Adobe Photoshop or other digital darkrooms are adjusted in the following order: auto-tone, desaturate, median filter (2 pixels), brightness/contrast (brightness −50, contrast +100), gradient map (tailoring is system specific), shadow/highlight (tailoring is system specific), glowing edges (edge width 3, edge brightness 20, smoothness 1), and then median filter (Radius 3 pixels). This system will increase the contrast of the roots against the agar media, while the glowing edges and final median filter make the root easier to track as it is larger.
10. ARTT software pre-tracking: Open the software from the shortcut on the desktop or start menu. Select the directory where your time-lapse series has been saved. You can choose which photos from the time series you measure using the drop-down menu. Go to the parameters tab, this has another sub-tab called settings that allows you to tell the software size of the root (preset to 50), the max orientation displacement (preset to 30) and maximum forward prediction (preset to 20). These values should be tailored to the distance your root tip was displaced which depends on its speed of growth and the length of time between each photo. We have never seen any effect associated with the Gaussian filter size so ignore that setting. Above this there is a tick box called “Apply inclusion

filter". If measuring the root tip we recommend this option as it stops the software measuring the movement of seeds or leaves. If measuring the Coleoptile tip, do not add the inclusion filter, this will cause the software to track all moving tips within the image series. There will be multiple moving parts tracked that will require discarding after the tracking has been completed, but this will allow you to select just the coleoptile for measuring. The measuring process in ARTT can take some time depending on the number of images in the time series but is usually really reliable and not prone to crashing.

11. ARTT software post-tracking analyses validation: After tracking has been completed, you should notice a drop-down menu labeled view in the top left hand corner; click on this and select analysis view. This will open a new window that will allow you to view the tracings made by the software. Reject any tracings the software made that are incorrect using the trace list in the bottom left hand corner, right click on the root and select delete. If two root paths cross each other the software can lose its tracking, but it may also regain it after the paths have crossed. If this occurs you can merge these two tracings into one by right clicking on the root name in the Trace list and selecting the merge option. If the software has lost the root tip you can move the tracking point by changing the X and Y values. If you are happy with your selection of roots then go to the top left corner and click on the single file option, this will ensure all the measurements are saved in one file instead of one file per root. Choose a destination directory and then click on the save data button.
12. Data processing in spreadsheet: Open the CSV data file, select all, copy and then paste it into Excel. This file contains the X and Y coordinates of the root tip, the time (which is the number of the image), how much it was displaced with its velocity, direction and orientation. The time column is actually the image number so should be converted to minutes, hours and/or days depending on the time scale of the experiment. This can be done by calculating the length of time between the first 2 photos and then extrapolating the rest of the time points. Displacement in ARTT is measured in pixel so should be converted to mm or cm. The accumulative displacement can be used to give you the root growth kinetics; this is achieved by adding together all the measurements from previous images. When the software fails to measure the root tip orientation it delivers either a "0" or "nan", these should be removed to prevent them skewing the final results. The root tip orientation can have the mean and standard error calculated averages and standard errors of each mutant/ecotype/treatment can be calculated and plotted on a graph.

References

1. Galvan Ampudia C, Julkowska M, Darwish E, Gandullo J, Korver R et al (2013) Halotropism Is a response of plant roots to avoid a saline environment. Curr Biol 23:204450
2. Massa G, Gilroy S (2003) Touch modulates gravity sensing to regulate the growth of primary roots of *Arabidopsis thaliana*. Plant J 33:43545
3. Takahashi N, Yamazaki Y, Kobayashi A, Higashitani A, Takahashi H (2003) Hydrotropism interacts with gravitropism by degrading amyloplasts in seedling roots of *Arabidopsis* and radish. Plant Physiol 132(805):10
4. Baldwin K, Strohm A, Masson P (2013) Gravity sensing and signal transduction in vascular plant primary roots. Am J Bot 100(1):126–142
5. Strohm A, Baldwin K, Masson P (2012) Molecular mechanisms of root gravity sensing and signal transduction. Wiley Interdiscip Rev Dev Biol 1:276–285
6. Strohm A, Baldwin K, Masson P (2012) Multiple roles for membrane-associated protein trafficking and signaling in gravitropism. Front Plant Sci 3:274
7. Larsen P (1962) Orthogravitropism in roots. In: Ruhland W (ed) Encyclopedia of plant physiol, Vol 17, Part 2, physiology of movements. Springer-Verlag, Berlin, pp 153–199
8. Perbal G, Jeune B, Lefranc A, Carnero-Diaz E, Driss-Ecole D (2002) The dose-response curve of the gravitropic reaction: a re-analysis. Physiol Plant 114:336–342
9. Young L, Harrison B, Murthy N, Moffatt B, Gilroy S, Masson P (2006) Adenosine kinase modulates root gravitropism and cap morphogenesis in *Arabidopsis thaliana*. Plant Physiol 142:564–573
10. Brunoud G, Wells DM, Oliva M, Larrieu A, Mirabet V, Burrow AH, Beeckman T et al (2012) A novel sensor to map auxin response and distribution at high spatio-temporal resolution. Nature 482(7383):103–106
11. Brooks T, Miller N, Spalding E (2010) Plasticity of *Arabidopsis* root Gravitropism throughout a multidimensional condition space quantified by automated image analysis. Plant Physiol 152(1):206–216
12. Wells D, French A, Naeem A, Ishaq O, Traini R, Hijazi H, Bennett M, Pridmore T (2012) Recovering the dynamics of root growth and development using novel image acquisition and analysis methods. Philos Trans R Soc B: Biol Sci 367(1595):1517–1524
13. Sangari A, Ohme-Takagi M, Mitsuda N, Assadi A (2012) Automated imaging & high-throughput phenotyping, massively parallel image acquisition, analysis and modeling: mapping plant genotype-phenotype relation. Cold Spring Harbor, Cold Spring Harbor, NY
14. Russino A, Ascrizzi A, Popova L, Tonazzini A, Mancuso S, Mazzolai B (2013) A novel tracking tool for the analysis of plant-root tip movements. Bioinspir Biomim 8:025004
15. Buer CS, Wasteneys GO, Masle J (2003) Ethylene modulates root-wave responses in *Arabidopsis*. Plant Physiol 132:1085–1096

http://www.springer.com/978-1-4939-3354-9

Environmental Responses in Plants
Methods and Protocols
Duque, P. (Ed.)
2016, XIV, 387 p. 79 illus., 58 illus. in color., Hardcover
ISBN: 978-1-4939-3354-9
A product of Humana Press