Protocol
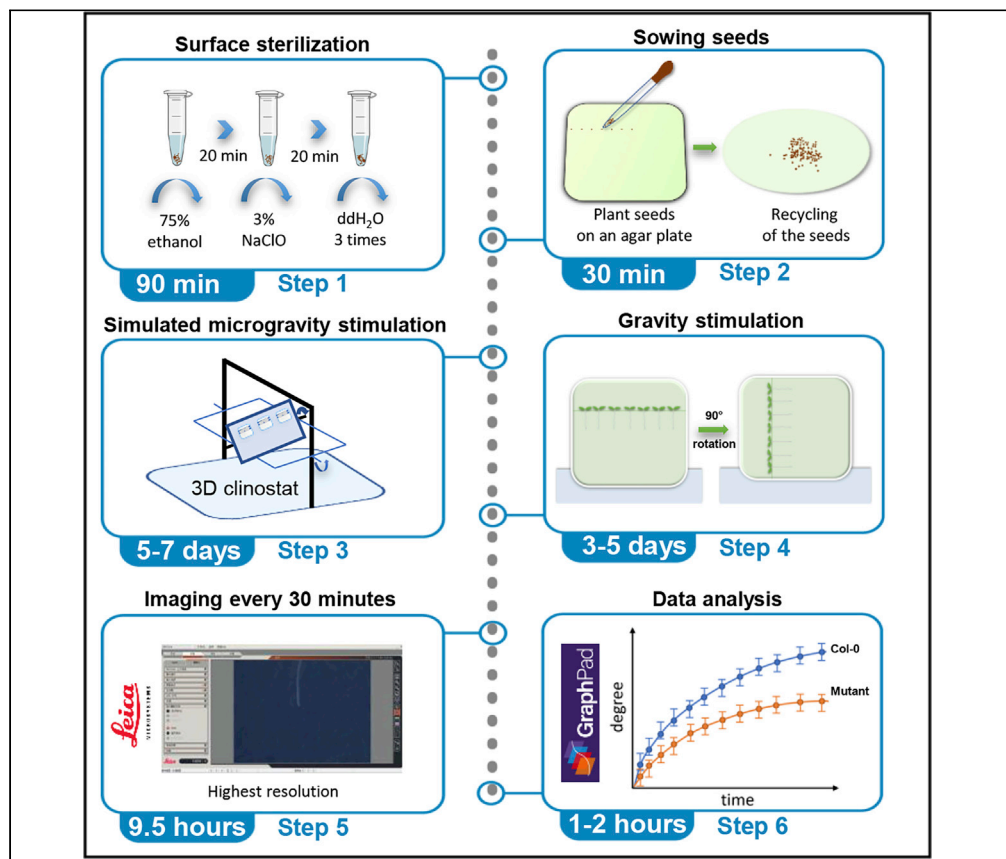
A protocol for measuring the response of *Arabidopsis* roots to gravity and treatment for simulated microgravity

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Highlights

Time-course quantification of root gravitropic response

Protocol for analyzing roots with indistinguishable gravitropic phenotype

Simulation of microgravity environment on the earth 1 g condition using a 3D clinostat

Applicable to analyze genes related to sensing or response to gravity/microgravity

We present a protocol to quantify the response of both normal and mutant *Arabidopsis* seedlings to gravity and simulated microgravity under earth-normal gravity conditions. We describe the steps to simulate microgravity using a three-dimensional (3D) clinostat, which changes the rate and direction at random and consistently rotates the axis horizontally and vertically to counteract the standard gravity at the Earth's surface. We then detail the gravity stimulation experiment, followed by the assessment of root responses using ImageJ-based analysis.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

STAR Protocols

Protocol

A protocol for measuring the response of *Arabidopsis* roots to gravity and treatment for simulated microgravity

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SUMMARY

We present a protocol to quantify the response of both normal and mutant *Arabidopsis* seedlings to gravity and simulated microgravity under earth-normal gravity conditions. We describe the steps to simulate microgravity using a three-dimensional (3D) clinostat, which changes the rate and direction at random and consistently rotates the axis horizontally and vertically to counteract the standard gravity at the Earth's surface. We then detail the gravity stimulation experiment, followed by the assessment of root responses using ImageJ-based analysis.

For complete details on the use and execution of this protocol, please refer to Xu et al. (2022).[1]

BEFORE YOU BEGIN

The protocol below describes the specific steps for analyzing the response of *Arabidopsis* roots to gravity and microgravity. However, we have also used this protocol (after slight modification) to analyze the response of the *Arabidopsis* hypocotyl and shoot.

Prepare solutions for surface sterilization of *Arabidopsis* seeds

⏱ Timing: 30 min

1. Prepare 75% (v/v) ethanol for the first step of surface sterilization of the *Arabidopsis* seeds. You can also buy the 75% (v/v) ethanol directly from commercial suppliers.
   a. Add 750 mL 100% ethanol to a 1,000 mL graduated flask.
   b. Add ddH$_2$O to the graduated flask to adjust the volume to 1,000 mL.
   c. Transfer the 1,000 mL 75% (v/v) ethanol into a 1,000 mL glass bottle with a cap so that it can be stored at room temperature for 3 months on the laboratory work bench.
2. Prepare 3% (v/v) NaClO for the second step of the surface sterilization of *Arabidopsis* seeds in the fume hood.
   a. Add 30 mL 100% NaClO solution to a 1,000 mL graduated flask.
   b. Add ddH$_2$O to the graduated flask to adjust the volume to 1,000 mL.
   c. Transfer the 1,000 mL 3% NaClO into a glass bottle with a cap.

Note: The 100% stock NaClO solution should be stored in the chemical fume hood and the 3% NaClO working solution can be stored at room temperature for 6 months on the laboratory work bench for use.

3. Prepare sterile ddH$_2$O for washing *Arabidopsis* seeds.
   a. Pour 250 mL ddH$_2$O from the laboratory water into each of four 500 mL glass bottles.
   b. Close the lid of the bottle slightly when autoclaving; do not screw the cap too tightly at this stage.

Prepare $^1/_2$ Murashige & Skoog (MS) solid medium

⏲ Timing: 30 min

4. Prepare 1,000 mL $^1/_2$ MS medium suspension

   Note: This step involves preparing the solid $^1/_2$ MS medium for *Arabidopsis* seed germination and seedling growth. You can use the medium directly after autoclaving when the medium is still in the liquid state. Alternatively, you can store it at 4°C for future use, microwaving or re-autoclaving it to melt prior to use. We strongly recommend ordering the MS powder directly from the commercial supplier instead of preparing different stock solutions of the MS medium components by oneself. The recipe of $^1/_2$ MS is listed in the table below.

   a. Put a 1,000 mL beaker onto a magnetic stirrer fitted with a hot plate and place a magnetic stirrer bar (e.g., 35 mm × 8 mm) into the beaker.
   b. Measure 2.203 g MS powder and 10 g sucrose and transfer them into the 1,000 mL beaker.
   c. Add approximately 980 mL ddH$_2$O to the beaker. Completely dissolve the MS powder and sucrose into ddH$_2$O with the aid of the hot plate (if necessary) and the magnetic stirrer.
   d. Adjust the pH to 5.8 slightly with NaOH solution, remove the stirrer bar and adjust the final volume to 1,000 mL.
5. Dispense the 1,000 mL $^1/_2$ MS solution into autoclavable glass bottles with screw caps.
   a. Measure 2.5 g agar powder (for bacterial culture medium) into each of four 500 mL glass bottles.
   b. Dispense 250 mL $^1/_2$ MS medium into each of the four bottles.
   c. Screw on the cap of the bottle loosely prior to autoclaving.

   ⚠ CRITICAL: In order to ensure the normal growth of *Arabidopsis thaliana*, it is necessary to keep the pH value of MS medium in the range of 5.7–5.8.

Step 5: We only half-fill the glass bottles with $^1/_2$ MS solid medium so that the medium does not spill out when re-melted by re-autoclaving or heating in the microwave oven. The $^1/_2$ MS solid medium can be stored at 4°C up to 2 weeks.

Prepare sterilized instruments and tools for seed handling in the laminar air flow clean bench

⏲ Timing: 4 h

6. Put 10 rubber pipette bulbs and 4 metal tweezers in a 500 mL glass beaker and cover the beaker opening tightly with aluminum foil and autoclave tape for autoclaving.
7. Put an appropriate number of 1.5 mL Eppendorf tubes into another 500 mL glass beaker up to about 3/4 of the beaker volume and cover it tightly with aluminum foil and autoclave tape for autoclaving.
8. Prepare at least 2 boxes of 1 mL pipette tips (P1000) for autoclaving.
9. Put at least 20 pieces of 18.5 cm diameter filter paper disks into a glass petri dish for autoclaving.
10. Prepare at least 4 Eppendorf tube racks with aluminum foil for autoclaving.

11. Fill an entire 500 mL beaker with cotton balls, pour 75% ethanol into the beaker till the cotton balls are fully soaked and place it onto the laminar air flow clean bench.
12. Prepare an ethanol lamp and put it and plastic sleeves of sterile experimental square plates onto the clean bench under the laminar air flow hood; wipe the outer surface of the plastic sleeves with 75% ethanol.
13. Put all the tools and tubes into the autoclave for the first cycle autoclaving. Start autoclaving (20 min at 15 psi at 121°C); at completion, wait until the temperature has cooled down to 60°C before emptying the autoclave.
14. Put ddH$_2$O and $^1/_2$ MS solution with agar from the above steps for the second cycle autoclaving, and the setting of the autoclave follows the step 13.
15. Transfer the sterilized ddH$_2$O, agar solution, instruments, and tools to the laminar air flow clean bench.

Prepare $^1/_2$ MS agar plates for *Arabidopsis* seed germination

⏱ Timing: 60 min

16. Pour the molten $^1/_2$ MS medium with agar into the experimental square plates (10 cm × 10 cm) when the temperature of the agar has cooled down to about 60°C and dispense 40–50 mL medium into each plate for seed germination and seedling growth studies.
17. Cover the lid incompletely and keep a sizable gap to allow the medium to be exposed to the air under the laminar air flow hood so that the inner surface of the lid is cleared of condensation, which would affect future observations.
18. Leave the plates on the clean bench until the medium solidifies.

Note: The plates can be immediately used. Alternatively, collect the plates, transfer them to the original plastic sleeve and move them to storage at 4°C for future usage and the plates can be stored at 4°C up to 2 weeks.

KEY RESOURCES TABLE

REAGENT or RESOURCES	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Murashige & Skoog Basal Medium with vitamins (MS)	Phytotech	Product ID: M519
Agar for bacterial culture medium	Sinopharm Chemical Reagent	Cat#10000561
Sodium hypochlorite solution	Sinopharm Chemical Reagent	Cat#80010428
Ethanol absolute	Sinopharm Chemical Reagent	Cat#10009218
Ethanol 75%	Sinopharm Chemical Reagent	Cat#80176961
Sucrose	Sinopharm Chemical Reagent	Cat#10021418
Experimental models: Organisms/strains		
Arabidopsis thaliana: Col-0	Arabidopsis Biological Resource Center (ABRC)	CS70000
Arabidopsis thaliana: *lazy4*(Col-0)	Yoshihara, T., and Spalding, E.P.[2]	SAIL_723_H11
Arabidopsis thaliana: *lazy2,3,4*(Col-0)	Ge, L., and Chen, R[3]	N/A
Software and algorithms		
ImageJ	Schneider et al.[4]	https://imagej.nih.gov/ij/
GraphPad Prism 8	GraphPad Software Inc	https://www.graphpad.com/scientific-software/prism/
Leica Application Suite Version 4.5.0 [Build: 418]	Leica Microsystems (Switzerland) Limited.	https://www.leica-microsystems.com
Other		
Petri dishes	Oiviass	Cat#PYM100FG
EP tubes	Axygen	Cat#MCT-150-C
Magnetic stirrer with heater	Dryer technology	Cat#ZNCL-T/ZNCL-TS

Continued

REAGENT or RESOURCES	SOURCE	IDENTIFIER
Autoclave	Panasonic	Cat#MLS-3751-PC 50L
Light chamber	Ningbo Southeast Instrument Co., Ltd	Cat#GXZ-100A
Optical microscopy	Leica Microsystems	Leica S8 APO
3D clinostat	National Space Science Center, Chinese Academy of Sciences	SM-31

MATERIALS AND EQUIPMENT

The 3D clinostat (SM-31 tow-axis driving clinostat) was designed and constructed by National Space Science Center, Chinese Academy of Sciences. The axis and frame of 3D clinostat was constructed by the material of aluminum alloy. Light source in the machine is from Light Emitting Diode (LED) which was fixed on one end of the inner frame with the light intensity of 6216 lx and ratio of R/B (red light / blue light) of 0.97. The rotating parameter of the 3D clinostat of both frames is from -1 Rpm (Round per minute) to 1 Rpm randomly.

Recipe of the $^1/_2$ MS agar medium	
Formula	mg/L
Ammonium Nitrate	825
Boric Acid	3.1
Calcium Chloride, Anhydrous	166.1
Cobalt Chloride·6H_2O	0.0125
Cupric Sulfate·5H_2O	0.0125
Na_2EDTA·2H_2O	18.63
Ferrous Sulfate·7H_2O	13.9
Magnesium Sulfate, Anhydrous	90.35
Manganese Sulfate·H_2O	8.45
Molybdic Acid (Sodium Salt)· 2H_2O	0.125
Potassium Iodide	0.415
Potassium Nitrate	950
Potassium Phosphate, Monobasic	85
Zinc Sulfate·7H_2O	4.3
Glycine (Free Base)	1
myo-Inositol	50
Nicotinic Acid (Free Acid)	0.25
Pyridoxine·HCl	0.25
Thiamine·HCl	0.05
Agar	10,000

STEP-BY-STEP METHOD DETAILS

Surface sterilization of *Arabidopsis thaliana* seeds in the laminar flow hood

⊙ Timing: 60 min

Surface sterilization of the seeds is critical for the experiment because the seeds need to germinate, and the seedlings grow on sterile $^1/_2$ MS agar plate solidified with agar.

1. Select the control and candidate mutant genotypes for gravity and simulated gravity response analysis.
   a. Transfer 100–200 *Arabidopsis* Col-0 seeds as the control from the seeds stock tube to a sterile 1.5 mL Eppendorf (EP) tube on the clean bench and mark the genotype name on the cover of the tube.

   b. Transfer 100–200 mutant seeds of *lazy2,3,4* triple mutant seeds into two separate sterile 1.5 mL EP tubes on the clean bench and mark their respective names on the tube covers.

2. Surface sterilization of the candidate seeds.

   a. Switch on the UV lamp in the laminar air flow hood over the clean bench and leave it on for 20 min.

   b. Pipette 1 mL 75% ethanol into each of the three 1.5 mL EP tubes and close the lids immediately.

   c. Shake the EP tubes vigorously for 10 s and then put them flat on the clean bench so that the seeds in the tube could be distributed evenly and completely soaked in the 75% ethanol solution.

Note: During the 20 min sterilization period, you can repeat shaking the tubes several times. Alternatively, you can put the tubes on a laboratory orbital shaker for 20 min.

   d. Put the EP tubes containing the seeds into a tube rack and let them stand for 1 min so that the seeds can settle down to the bottom of each tube.

   e. Remove as much of the 75% ethanol as possible with a 1 mL automatic pipette without aspirating any seeds in the tube.

Note: A small amount of the 75% ethanol remaining in the tube is acceptable.

   f. Pipette 1 mL 3% NaClO into each of the EP tubes and repeat step c above.

   g. Put the three EP tubes into the tube rack and take them to the laminar air flow clean bench. Before opening the bench, make sure that the UV lamp is turned off.

  ⚠ CRITICAL: Note that it is difficult to precisely count the number of seeds, but more seeds are better than insufficient seeds and we have a method to recycle any extra seeds. If your mutant is not Col-0 (e.g., Ler-0) background, please use the wild type with the same background (Ler-0) as the control.

Wash seeds with sterilized ddH$_2$O

⏱ Timing: 30 min

The goal of this step is to completely remove the remaining ethanol and NaClO and replace them with sterilized ddH$_2$O so that the seeds can germinate normally.

3. Replace the 3% NaClO in the EP tube with sterilized ddH$_2$O.

   a. Remove as much of the 3% NaClO as possible with a 1 mL automatic pipette without aspirating seeds.

   b. Add 1 mL sterile ddH$_2$O into the tube, shake the tube and leave it to stand for 1 min until the seeds have completely settled down to the bottom.

   c. Repeat step b above two more times, adding only 500 μL sterilized ddH$_2$O in the tube at the last rinse.

4. When the seeds have completely settled, you can proceed to the next step, or store these seeds in the ddH$_2$O at 4°C for up to one week in the dark.

  ⚠ CRITICAL: Make sure that the NaClO and ethanol are removed completely; any traces remaining can affect the seed germination. The final 500 μL aliquot of sterile ddH$_2$O can be replaced with 500 μL 0.1% agar (sterilized) so that the seeds can be suspended and can be easily dispensed onto the grown medium.
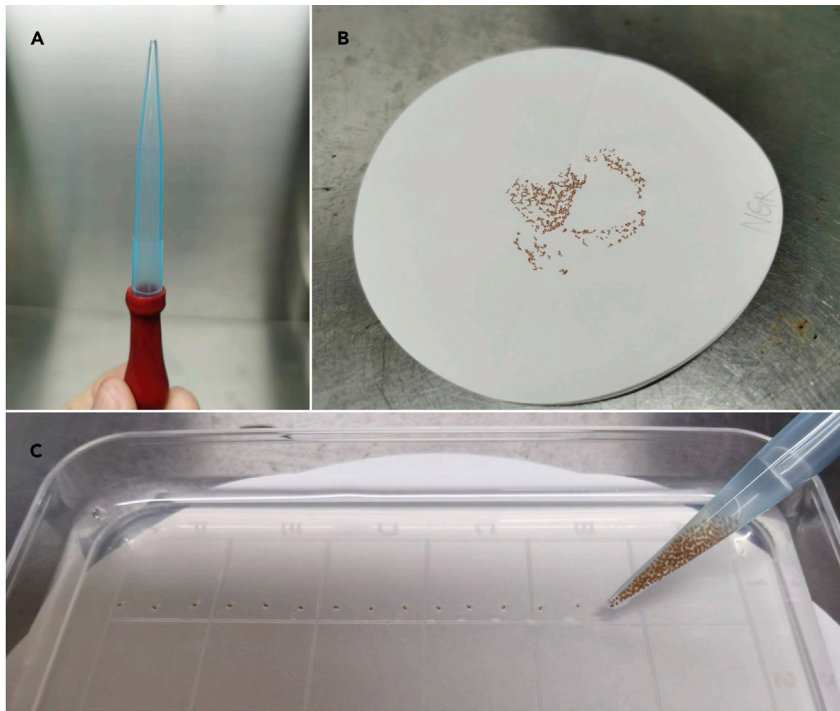
Figure 1. Sowing seeds onto a $^1/_2$ MS agar plate and recycling of the surface-sterilized *Arabidopsis* seeds
(A) Attachment of the rubber pipette bulbs on the 1 mL pipette.
(B) Recycling of the additional seeds on a filter paper.
(C) Sowing seeds on a $^1/_2$ MS agar plate.

Plant seeds on $^1/_2$ MS agar plate

Ⓣ Timing: 30 min

Different researchers have their own methods to sow the tiny *Arabidopsis* seeds onto a $^1/_2$ MS agar plate. We have developed a highly efficient method which can save a lot of time.

5. Attach a rubber pipette bulb firmly to the top of a 1 mL pipette tip (Figure 1A).
6. Open the EP tube containing the surface-sterilized seeds and take up about 200 μL ddH$_2$O with adequate number of seeds in the pipette tip. Hold the pipette tip vertically until the seeds settle down to the narrow opening of the pipette tip.
7. Open the cover of the agar plate containing $^1/_2$ MS agar and touch the surface of the medium slightly with the pipette tip containing the seeds so that the seeds can be expelled together with the ddH$_2$O onto the medium.
   a. Use the rubber bulb to balance the force in the vertical direction to control the number of the seeds delivered in each droplet until one droplet only contains one *Arabidopsis* seed (Figure 1C).
   b. For gravity response analysis, plant about 20 seeds of Col-0 on one plate and 20 seeds of the mutant on another plate respectively.
   c. For simulated microgravity analysis, sow 6–7 seeds of Col-0, *lazy4*, and *lazy2,3,4* triple mutant respectively on the same plate.

   Note: Two plates are required here (one for simulated microgravity and another for the control).

8. Leave the plate with the cover half-off in the laminar air flow clean bench for 20 min until the ddH$_2$O around the seeds has evaporated.

9. Cover the plates and seal them with micropore.
10. Stratify the plates at 4°C for 3 days in the dark.
11. For the seeds remaining in the EP tubes, we can absorb the remaining water from the seeds in the pipette tip by dispensing them completely onto a sterile filter paper disk.

   Note: Dry the paper with seeds at room temperature and mark the name of the seeds on the paper with a pencil (Figure 1B); these seeds can be stored in this form at 4°C in a sterilized petri dish for 2 months. Alternatively, the seeds can be returned to a sterile EP tube.

   ⚠ CRITICAL: Step 7 can be challenging for the novice but practice will significantly improve your skill and ability. Remember to change to a new pipette tip when you start dispensing seeds of a new genotype. Make sure that the seeds at 4°C are stored in absolute darkness, otherwise the seeds will germinate sooner. The filter paper with the seeds attached can be stored in a glass petri dish at 4°C after the seeds and paper have completely dried.

3D clinostat-based simulated microgravity analysis in a plant growth room

🕓 Timing: 5–7 days

All the experiments carried out on Earth's surface are consistently exposed to the standard gravity Earth 1 g condition. While it is impossible to create a microgravity environment on the Earth, but microgravity can be simulated through continuous changing of the object's orientation relative to the gravity vector through 3D clinostat. Such simulation can generate effects comparable to true microgravity when the change is faster than the response time of the object to gravity. The 3D clinostat has two independent rotating frames that are set perpendicular to one another. Such a machine can simulate a microgravity environment based on the gravity vector averaging zero by continuously rotating the axis. Simulated microgravity analysis begins from the seed germination stage to 5 days or even more, while gravity response analysis begins when the roots of the seedlings have grown to about 2 cm in length in the light chamber. Here, we use the Col-0 wild type, *lazy4*, and *lazy2,3,4* triple mutant for the simulated microgravity analysis.

12. Fix the Col-0, *lazy4*, and *lazy2,3,4* triple mutant plates to the 3D clinostat and control equipment, respectively, in the same plant growth room after 3 days of stratification (Figures 2A and 2B).
13. Start the 3D clinostat program: 16 h light and 8 h dark; the sample stage was three dimensionally rotated by changing the rate and direction of rotation at random from 1 to −1 (reverse direction) rpm every 1 min[5] (Figure 2C).
14. Check the growth of the seedlings and take photos on the 3rd, the 5th, or the 7th day.

   ⚠ CRITICAL: Check on the plates on the 3D clinostat and make sure that the machine is working well every day. Taking pictures of the seedlings on the 3D clinostat should be quick. Make sure to return the plates to the 3D clinostat as soon as possible after finishing the imaging.

Seed germination and growth in the light chamber for gravity stimulation analysis

🕓 Timing: 3–4 days

15. Move the plates from the 4°C refrigerator to a plant growth chamber (21°C, 16 h light and 8 h dark) and incubate them vertically for 3–4 days.
16. Exchange the position of the plates in the plant growth chamber every day at the same time of day until the roots of the seedlings have grown to about 2 cm to avoid any position effect due to uneven light intensity.
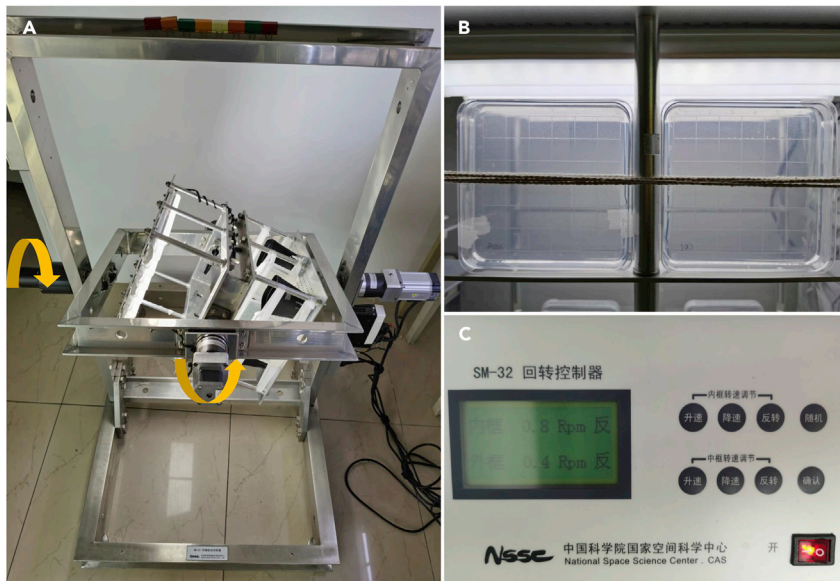
Figure 2. 3D clinostat-based simulated microgravity analysis
(A) An overview of the 3D clinostat. The two perpendicular axes rotate independently when it is working. Col-0, *lazy4*, and *lazy2,3,4* triple mutant plates were fixed to the 3D clinostat under light.
(B) The ground 1 g control plates are in the same room with 3D clinostat.
(C) The control panel of the 3D clinostat. It showed the inner frame of 0.8Rpm and the outer frame of 0.4 Rpm instantaneously.

Gravity stimulation experiment

⏱ Timing: 9.5 h

The shoot endodermis and root columella are the major gravity-sensing tissues in plants. Their statocyte cells (containing amyloplast-statoliths) function as gravity sensors that play a vital role in both shoot and root gravitropic growth. Changing of the gravity vector can stimulate the plant to adapt to the new vector through gravitropic shoot and root growth. Since the gravity vector is consistent and pointing to the ground direction, we can manually rotate the growth direction of the *Arabidopsis* roots by 90° relative to the gravity vector to achieve gravity stimulation.

17. Select 6–7 well-grown seedlings of Col-0 and mutant and carefully transfer them from the original plates to a new plate in the clean bench.
18. Incubate the seedlings in the growth chamber for 3 h.
19. Rotate the new plate to 90° in the chamber without altering the other conditions.
20. Observe the root growth and bending conditions by optical microscopy and take photos every 30 min, using the Leica imaging software (Leica Application Suite).
    a. Open the Leica imaging software and observe the live condition of the root through the "Acquire" button and adjust the focus and light intensity to obtain a clear image of the root (Figure 3).
    b. Use the highest resolution available (e.g., 2592 × 1944) on the "Format" button on your own software to capture the image.
    c. Click on the button of "Capture Image" and the image will be stored automatically in the designated file.

Note: The 3–4 day old *Arabidopsis* root is in the center of the view. Adjust the focus so that the outline of the root is clear enough for observation and capturing image. Remember to use the highest resolution available when capturing image.
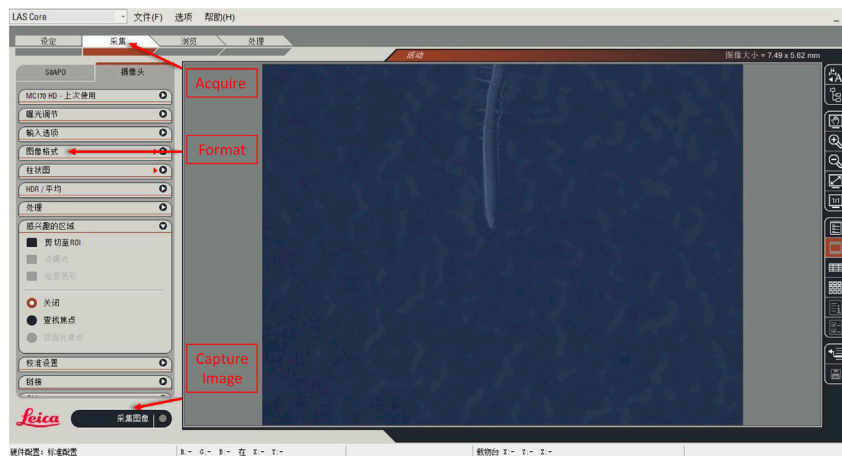
Figure 3. Leica software of the optical microscopy for root imaging

Data analysis of the time-course-based root gravity sensing

⏱ **Timing: 2 h**

The *Arabidopsis* root will bend quickly after sensing the new gravity vector after 90° rotation. Manually measuring the bending angle is not only laborious but also error-prone. ImageJ-based (https://imagej.nih.gov/ij/) analysis of the bending angle provides a better solution for precisely quantifying the data. Usually, the angle between the root-bending direction and the horizontal line can be defined as the bending angle "α" (Figure 4).

Make a full circle in the columellar region and let the lateral root cap be a tangent to the full circle, and then draw a line linking the center of the circle and the root tip. Alternatively, you can draw two lines that are tangential to the lateral root cap on each side, linking the intersection of the two lines with the root tip to form another line. This line forms an angle "α" with the horizontal line and the angle "α" is the bending angle of the root after gravity stimulation (also see Figure 6).

21. Measure the angle of the root growth direction against the horizontal line (the former growth direction) using the ImageJ software.
    a. Open the software, "File" → "Open" → choose the root picture (Figure 5).
    b. Make the picture full screen and use the "Magnifying glass" button or "Shift" + "+" button to magnify the root tip region for more accurate measurements. Use the "Scrolling tool" to keep the root tip in the central position.
    c. Use the "oval" tool to make a full circle (press "shift" simultaneously) in the columellar region and let the lateral root cap be a tangent to the full circle.
    d. Draw a line linking the center of the circle and the root tip.

    Note: This line forms an angle "α" with the horizontal line and the angle "α" is the bending angle of the root after gravity stimulation (Figures 4 & 6). Alternatively, you can draw two lines that are tangential to the lateral root cap on each side, linking the intersection of the two lines with the root tip to form another line.

    e. Use the "Angle tool" to follow the track of horizontal line and the bending direction line and then click "Analyze" on the main menu and then "Measure" in the drop-down menu to measure the angle "α" and keep the record of gravity stimulation for each time-course of both Col-0 and the mutant.

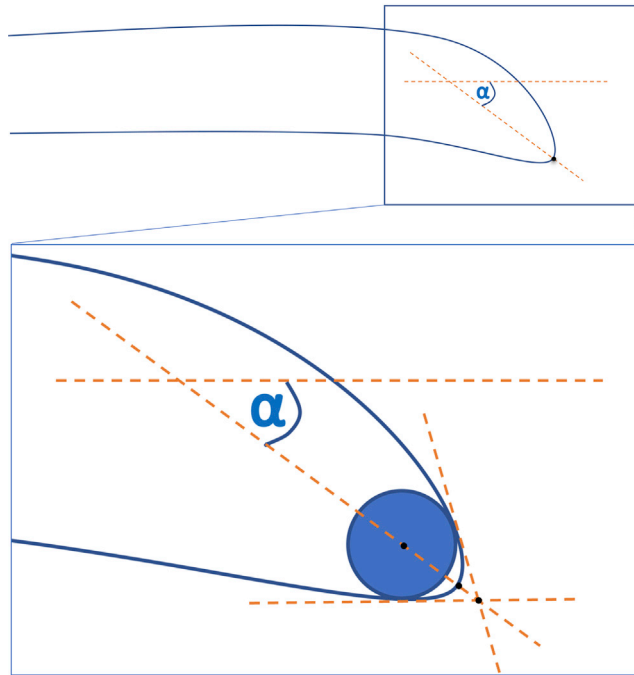**Figure 4. The definition of the root-bending angle, α**

22. GraphPad Prism 8-based statistical analysis of the root gravity response.
    a. Open the software and choose "Grouped" → "Enter or import data into a new table" → "Enter 6 replicate (usually at least 3 replicates) values in side-by-side subcolumns" → "Create" (Figure 7).
    b. Define the "Group A" as "Col-0" and "Group B" as "Mutant". Define the "Title" as time course from "30 min" to "360 min" with intervals of 30 min (Figure 8).
    c. Fill in the blank with the data measured by the ImageJ software and then click "Data 1" in the sub-menu of "Graphs" on the left menu (Figure 8).
    d. The "Change Graph Type" slide then appears a new window (Figure 9); choose "Grouped" under "Graph family" option.
    e. Choose "Superimposed symbols (at mean or median) with connecting line" under the "Summary data" option.
    f. At last, choose "Mean with SD" under "Plot" option and click "OK" when complete.
    g. The software will automatically generate a chart of the time-course analysis of the root-bending angle in response to gravity stimulation (Figure 12).
    h. Rename the X axis and the Y axis, if necessary. Use "Change Colors" button on the main menu to generate different colors of the samples. Alternatively, "Black and White" will be adopted for the default setting.
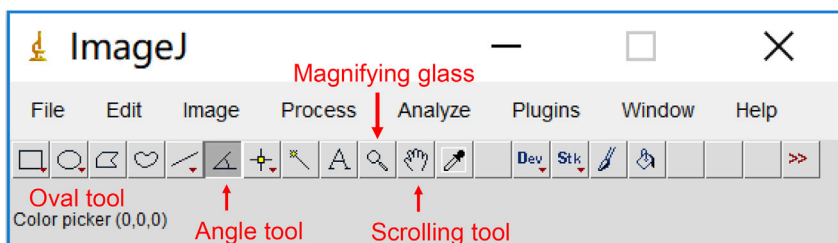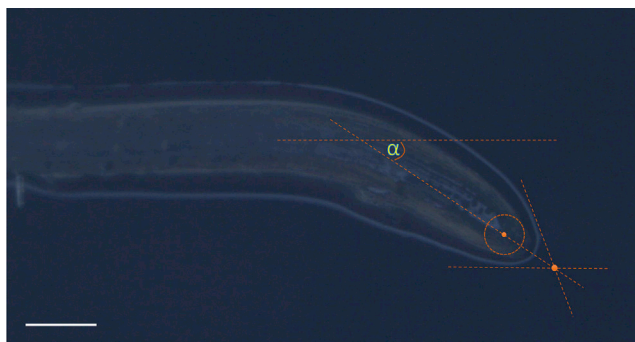

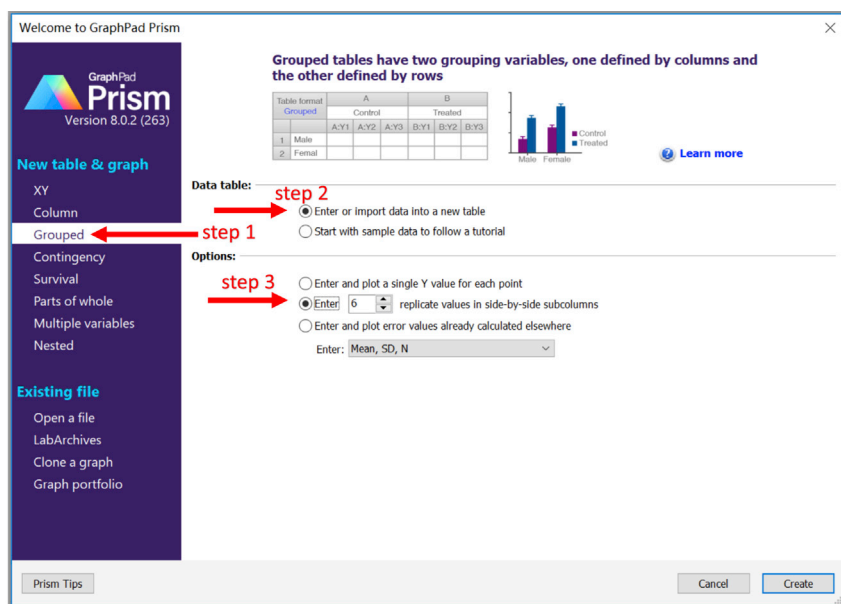**Figure 5. ImageJ-based measurement of the root-bending angle**

Figure 6. Measurement of the bending angle with the auxiliary line
Bar = 100 μm.

EXPECTED OUTCOMES

For the gravity response analysis, if the mutant affects the root gravity response or sensing, the root-bending angle of the mutant will show a significant difference from that of the wild-type Col-0 after 6 h of gravity stimulation through a 90° rotation. Otherwise, there will be no significant difference compared with Col-0. We observed the root-bending angle of Col-0 and the mutant by optical microscopy and took photos every 30 min (Figure 10).

For the simulated microgravity response analysis, we take photos every day, and the photos taken on the 3rd, 5th or 7th days and the responsive phenotypes are usually analyzed and compared with the standard gravity ground control under the same growth conditions (Figure 11). However, it is difficult to quantify the phenotype of the *Arabidopsis* root after the 3D clinostat treatment because of the phenotypic randomness. A number of techniques can be employed to analyze the tissue specific differences of the *Arabidopsis* treated by the simulated microgravity at the molecular level, such as RNA-seq and proteomics[6–8]; at the cellular level, such as immunofluorescence imaging and Scanning electron micrograph[9–11]; and at the physiological level, such as enzymatic activity analysis[1]; or even the single cell sequencing (no reports about the microgravity or simulated microgravity in plants).

Figure 7. Settings of the GraphPad Prism 8 for starting a new analysis

Figure 8. Re-define the group names and title names

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantification of the bending angle was conducted using the ImageJ and GraphPad Prism software as described above. The software will automatically generate a chart of time-course analysis of the root-bending angle in response to gravity stimulation (Figure 12).

LIMITATIONS

This protocol describes a method to analyze the response to gravity and simulated microgravity of a candidate mutant and its corresponding wild type. For mutants completely deficient in gravity-sensing ability, it is easy to observe the marked difference by comparing them with the wild-type control. However, many mutants do not show a significant phenotype and it can be difficult to distinguish the mutant from the wild-type control. Time-course analysis of the root-bending angle provides an appropriate parameter by which to analyze minor phenotypic alterations through quantifying the bending angle. Gravity response can be quantified directly through the time-course root bending angle analysis after 90° rotation. However, simulated microgravity response requires the collaboration with other techniques and subjects to quantify the variation through multiple dimensional level, such as mathematical modeling and live imaging. Although this protocol provides clear and detailed steps to observe and quantify the response to gravity, and treatment for simulated gravity, there are still some limitations that should be addressed and taken note of.

In this protocol, we use a 3D clinostat to simulate the microgravity environment. Although this method has been reported in many publications, it still cannot represent true microgravity, which can be obtained only during spaceflight or when an object is undergoing free-fall motion. Plants use the amyloplast-statoliths in the statocytes to sense gravity. 3D clinostat simulates the
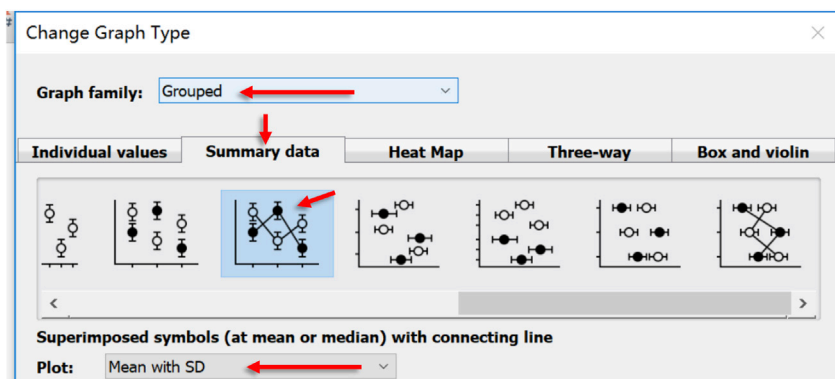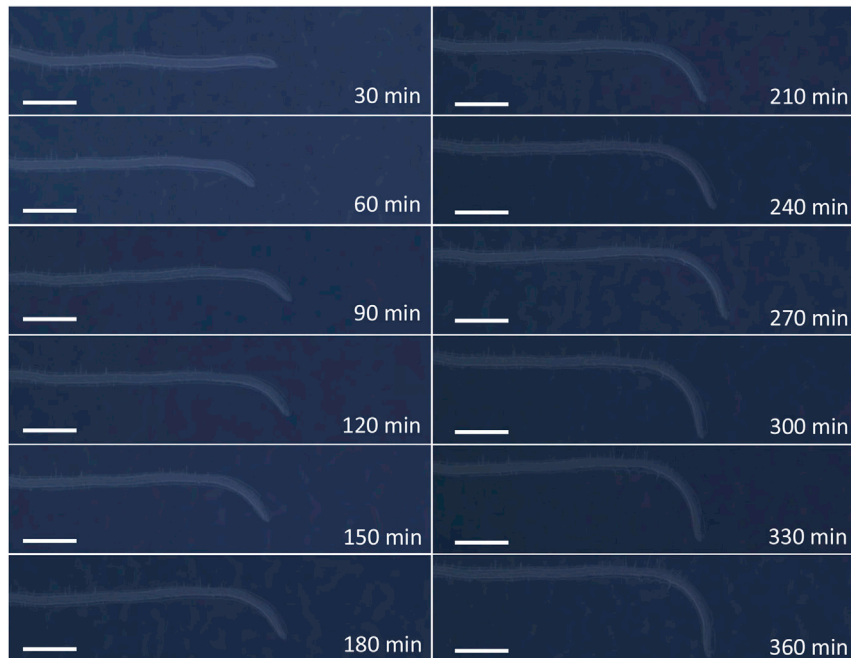

Figure 9. Settings for the graph type

Figure 10. Time-course analysis of changes in *Arabidopsis* Col-0 root-bending angle in response to 90° rotation
Bars = 500 μm.

microgravity environment on the earth 1 g condition based on the starch-statolith theory in plants. However, no sensing body that is responsible for gravity sensing has been reported in vertebrates and microorganisms, so 3D clinostat may be not suitable for vertebrate or microorganism research, although research has been published on its use in these organisms. Another limitation is that 3D clinostat derived data (root images) can't be easily analyzed and quantified by image capturing because of randomness of the root growth and bending direction caused by the randomly rotated two axes. Collaborations with scientists in other fields such as mathematics and machine learning may provide insights into analyzing and quantifying 3D clinostat-based data.

TROUBLESHOOTING
Problem 1
Low germination levels (steps 13 and 14).

Potential solution
Check the age of the seed stock as well as the environment under which the seeds are stored. Storing seeds at room temperature is the main reason for low germination levels. We recommend keeping the dried seeds in the desiccant containing EP tube at 4°C or −20°C in the refrigerator or domestic freezer, respectively.
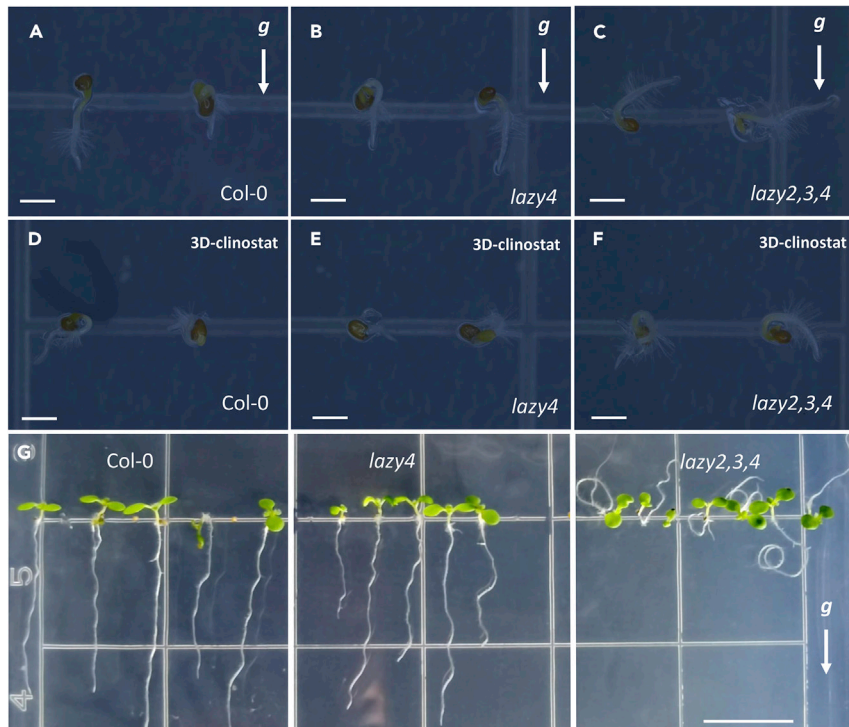
Problem 2
Seed coats become white and seeds germinate late on the agar plates (steps 13 and 14).

Potential solution
White seed coats are usually caused by the oxidation reaction of the NaClO. Please make sure to rinse away the 3% NaClO using sterile ddH$_2$O at least two times or even more.

Problem 3
Contamination on the $^1/_2$ MS agar plates (steps 13 and 14).

Figure 11. Root phenotypes of *Arabidopsis* Col-0 and the *lazy2,3,4* triple mutant on the 3rd and 7th day after simulated microgravity stimulation on a 3D clinostat

(A–F) Three-day old *Arabidopsis* Col-0 (A, D), *lazy4* (B, E) and *lazy2,3,4* (C, F) seedlings on earth-normal gravity condition (A–C) and 3D-clinostat (D–F).

(G) Seven-day old *Arabidopsis* seedlings grew on earth-normal gravity condition.

Bars = 1 mm (A–F), bar = 1 cm (G).

Potential solution

Make sure to switch on the UV lamp at least 20 min before sterilization and start the laminar flow motor before you start to plant seeds on the plate and use disposable gloves during the whole process of seed handling.

Problem 4

The root bending image is too blurry to draw the full circle and lines in the step 21 of the "step-by-step method details", in order to achieve measurement.

Potential solution

Make sure to select the highest resolution setting on the "Format" button.

Problem 5

Abnormal growth of some seedlings (steps 13 and 14).

Potential solution

Abnormal seedlings usually develop from incompletely matured seeds, or because the roots have sunk too deeply into the agar. Ignore such individual plants and do not include their data.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Dr. Peipei Xu; email: ppxu@cemps.ac.cn.
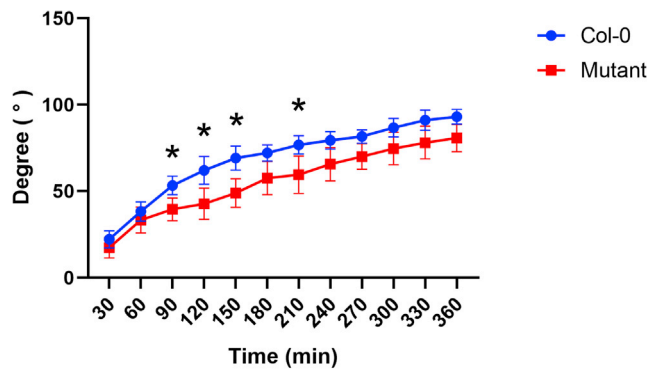
Figure 12. Time-course gravitropic response (mean ± SD) of the Col-0 and mutant root-bending angles after gravity stimulation

The symbol (*) shows significantly different between Col-0 and mutant (p value < 0.05) on the corresponding time point. Statistical significance determined using the Holm-Sidak method, with alpha = 0.05. Each row was analyzed individually, without assuming a consistent SD.

Materials availability

This study did not use novel reagents or plant materials. All reagents and *A. thaliana* lines are available from the previous publication.[2,3,12]

Data and code availability

The published article includes all datasets and codes generated during this study.

AUTHOR CONTRIBUTIONS

W.M.C., H.Q.Z., and P.P.X. designed the research, contributed to supervision and grant acquisition, and obtained the data; J.B.H. and C.H.Y performed the experiments and data analysis and wrote the original draft manuscript. All authors reviewed the final draft and approved submission.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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