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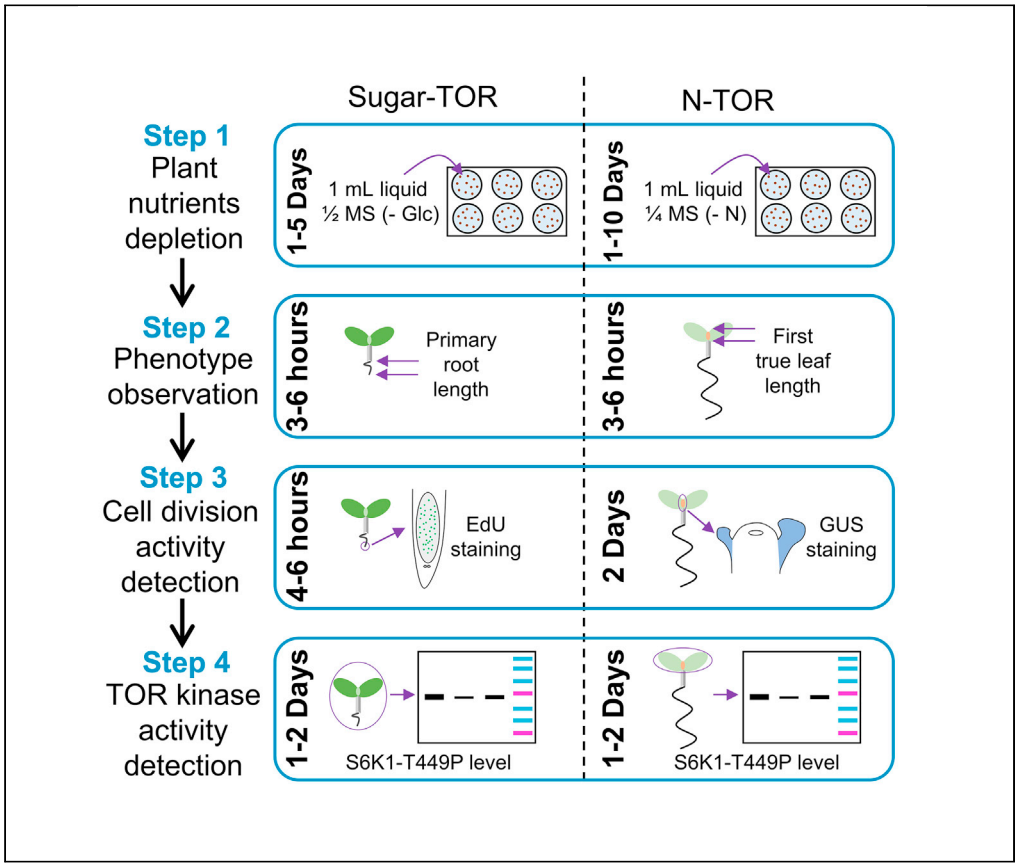
Protocol

Liquid culture system for efficient depletion of the endogenous nutrients in Arabidopsis seedlings



Target of rapamycin (TOR) is a central regulator in nutrient signaling. However, the innate capacity of autotrophic plants to produce carbon-related nutrients and nitrogen-related nutrients makes studying the TOR pathway difficult. Here, we describe a protocol for a liquid culture system for efficient depletion of the endogenous carbon/nitrogen nutrients in Arabidopsis seedlings. Exogenous carbon/nitrogen can be supplied to dissect the TOR pathway.

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Highlights

Liquid culture system for depletion of endogenous carbon/nitrogen in Arabidopsis

Exogenous carbon/nitrogen can be supplied to dissect the nutrient-TOR signaling

Nutrients regulated cell proliferation in root/shoot apex depends on TOR kinase

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Liquid culture system for efficient depletion of the endogenous nutrients in Arabidopsis seedlings

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SUMMARY

Target of rapamycin (TOR) is a central regulator in nutrient signaling. However, the innate capacity of autotrophic plants to produce carbon-related nutrients and nitrogen-related nutrients makes studying the TOR pathway difficult. Here, we describe a protocol for a liquid culture system for efficient depletion of the endogenous carbon/nitrogen nutrients in Arabidopsis seedlings. Exogenous carbon/nitrogen can be supplied to dissect the TOR pathway.

For complete details on the use and execution of this protocol, please refer to Xiong et al. (2013) and Liu et al. (2021).

BEFORE YOU BEGIN

Note: The liquid medium culture systems described in this protocol can generate stable mitotic quiescent seedlings to study the underlying mechanisms of nutrient-TOR signaling in the regulation of cell proliferation in the shoot and root apexes, and have been used to study the role of glucose-TOR signaling in root meristem cell proliferation (Fu et al., 2021; Xiong et al., 2013), and the role of nitrogen-TOR signaling in shoot apex cell proliferation (Liu et al., 2021). This protocol has broad utility and can also be applied, with some modifications, to study other molecular functions of plant nutrient signaling including cell elongation (Yuan et al., 2020), cell division interaction with environmental signals (Li et al., 2017), and metabolite mediated circadian regulation (Zhang et al., 2019).

Plant seed preparation

Timing: 2 d–5 d before the experiment

All *Arabidopsis* lines (WT, *35S::S6K1-HA*, *pCYCB1; 1::GUS*) used in this study are of the Columbia ecotype (Col-0) background. High quality seeds are essential for the protocols described below. We recommend using the same growth conditions for your plants and culturing and managing all of the *Arabidopsis* plants together, then harvesting the seeds at the same time. Please refer to the excellent protocols described in *Arabidopsis Protocols (Third Edition)* (Rivero et al., 2014), for detailed methods on planting, culturing and managing *Arabidopsis* plants, and harvesting and storing high-quality *Arabidopsis* seeds.

Arabidopsis seedlings need to be grown in a sterile environment and the seeds need to be surface-sterilized:

1. Seed surface sterilization.
   a. Prepare about 100 seeds in a 1.5 mL Eppendorf tube, add 1 mL ultrapure water and mix well. Put the tube upright at room temperature (23°C–25°C) for 10 min.
   b. Remove the water and add 1 mL 75% (v/v) ethanol. Gently mix on a shaker for 5–10 min.
   c. Discard the ethanol and wash with 1 mL sterile ultrapure water for 5 min. Repeat this step 3 times. This step should be performed on a clean benchtop.

   Note: Our laboratory uses 75% (v/v) ethanol for the seeds surface sterilization protocol. Other sterilizing methods (e g. treat the seeds with 5% (v/v) bleach for 5 min followed by 75% (v/v) ethanol treatment for another 2 min) can also be used.

2. Keep the surface-sterilized seeds in the tube with 1 mL sterile ultrapure water at 4°C in the dark for 2–5 d to promote uniformed germination prior to using the seeds in experiments.

Sugar-free and inorganic nitrogen-free liquid medium preparation

Timing: 1 h before the experiment

3. Sugar-free half strength Murashige and Skoog (1/2 MS) liquid growth medium:
   a. In 900 mL ultrapure water, dissolve 2.215 g MS salt (with vitamins), 1 g MES (final 0.1% (w/v)). Adjust pH to 5.7 using 5 M KOH. Add additional ultrapure water to bring the volume up to 1 L. Do not add any sugar or sugar-containing buffers.
   b. Filter sterilize. The medium can now be stored at room temperature (23°C–25°C) in the dark for up to 6 months.
   c. Aliquot 50 mL of 1/2 MS (sugar-free) in a 50 mL sterilized tube. This medium is ready for plant sugar depletion incubation.
   d. In 45 mL ultrapure water, dissolve 0.111 g MS salt (with vitamins), 0.05 g MES (final 0.1% (w/v)) and 0.25 g (final 0.5% (w/v)) glucose in a 50 mL tube. Adjust pH to 5.7 using 5 M KOH. Add additional ultrapure water to bring the volume to 50 mL, and filter sterilize. This glucose containing medium is used for positive control experiments. The use of glucose or sucrose as a sugar source has similar effects in this assay. For details, please refer to Xiong et al. (2013), Supplementary Figure 3.
4. Inorganic nitrogen-free quarter strength Murashige and Skoog (1/4 MS) liquid growth medium:
   a. In 900 mL ultrapure water, dissolve 0.8075 g MS inorganic nitrogen-free salt (with vitamins, 1/4 MS inorganic nitrogen-free medium) or 1.107 g MS salt (with vitamins, 1/4 MS inorganic nitrogen-containing medium), 1 g MES (final 0.1% (w/v)), 1.8 g glucose (final 10 mM or 0.18% (w/v)). Adjust pH to 5.7 using 5 M KOH. Add additional ultrapure water to bring to 1 L.
   b. Filter sterilize. The medium can now be stored at room temperature (23°C–25°C) in the dark for up to 6 months.

   Note: We use ultrapure water in the medium preparation. All containers should be washed 3 times by ultrapure water prior to use. Filter sterilization (using a 0.22 μm filter) of the prepared medium is highly recommended.

   Note: The difference between 1/4 inorganic nitrogen-free MS medium and 1/4 inorganic nitrogen-containing MS medium is that the former does not have any inorganic nitrogen and the latter contains inorganic nitrogen: nitrate and ammonium.

   Note: We recommend using 1/4 MS (inorganic nitrogen-free) liquid medium instead of 1/2 MS or full MS (inorganic nitrogen-free) medium together with 0.18% (w/v) glucose instead of 0.5%

(w/v) glucose to generate inorganic nitrogen depletion seedlings since high strength MS medium or a high concentration of glucose may cause nutrients ratio unbalancing, leading to undesired stress-related phenotypes.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal S6K1 p-T449 (1:2,000)	Agrisera	Cat#AS132664
Conjugated-HRP Anti-HA tag antibody (1:5,000)	Sigma-Aldrich	Cat#12013819001; RRID:AB_390917
Mouse monoclonal Anti-Tubulin (1:5,000)	Abmart	Cat#M30109
Anti-Rabbit IgG (whole molecule) -Peroxidase antibody (1:5,000)	Sigma-Aldrich	Cat#A0545
Anti-Mouse IgG (whole molecule) -Peroxidase antibody (1:5,000)	Sigma-Aldrich	Cat#A4416
Chemicals, peptides, and recombinant proteins		
Torin2	Tocris Bioscience	Cat#4248
X-Gluc	Sigma-Aldrich	Cat#B5285
Glucose	Sigma-Aldrich	Cat#G5767
KNO_3	Sigma-Aldrich	Cat#G8394
Ethanol	SCRC	Cat#10009218
Methanol	SCRC	Cat#10014108
MES	Sigma-Aldrich	Cat#M3671
KOH	SCRC	Cat#10017008
KCl	Sigma-Aldrich	Cat#P3911
KH_2PO_4	Sangon Biotech	Cat#A501211
NaCl	Sigma-Aldrich	Cat#S9888
Na_2HPO_4	Sangon Biotech	Cat#A501727
NaH_2PO_4	Sangon Biotech	Cat#A501726
potassium ferricyanide ($K_3[Fe(CN)_6]$)	SCRC	Cat#10016718
potassium ferrocyanide ($K_4Fe(CN)_6 \cdot 3H_2O$)	SCRC	Cat#10016816
EDTA	SCRC	Cat#10009617
Triton X-100	Sigma-Aldrich	Ca#X100
X-Gluc	Thermo Fisher	Ca#R0851
Tris-HCl	Diamond	Cat#A100234
SDS	SCRC	Cat#30166480
β-mercaptoethanol	SCRC	Cat#80076927
Glycerol	SCRC	Cat#10010618
Bromophenol blue	SCRC	Cat#71008080
Tween-20	SCRC	Cat#30189328
Formaldehyde	SCRC	Cat#100100008
BSA	Sigma-Aldrich	Cat#A1933
Lactic acid	Sangon Biotech	Cat#A501681
Click-iT EdU Alexa Fluor 488 HCS	Thermo Fisher	Cat#C10350
MS salt with vitamins	CAISSON LABS	Cat#MSP09
MS salt with vitamins, nitrate free	CAISSON LABS	Cat#MSP07
SuperSignal West Pico PLUS chemiluminescent substrate	Thermo Fisher	Cat#34578
Deposited data		
Original gel imaging data 1	This study, Mendeley	https://doi.org/10.17632/h8px3kzd6g.3
Original gel imaging data 2	This study, Mendeley	https://doi.org/10.17632/d2d9tpjtv4.1
Experimental models: Organisms/strains		
Arabidopsis: Col-0 (WT)	(Xiong et al., 2013)	CS1092
Arabidopsis: pCYCB1; 1::GUS	(Colon-Carmona et al., 1999)	N/A
Arabidopsis: 35S::S6K1-HA	(Xiong and Sheen, 2012)	N/A
Software and algorithms		
Image J	Image J v1.8.0	https://imagej.nih.gov/ij/

MATERIALS AND EQUIPMENT

Preparation of plant growth media

1/2 MS sugar-containing or sugar-free medium

Reagent	1/2 MS sugar-containing (mg/L)	1/2 MS sugar-free (mg/L)
Glucose	5000.0	0.0
MES	1000.0	1000.0
Potassium Nitrate (KNO_3)	950.0	950.0
Ammonium Nitrate (NH_4NO_3)	825.0	825.0
Calcium Chloride, Anhydrous ($CaCl_2$)	166.1	166.1
Magnesium Sulfate, Anhydrous ($MgSO_4$)	90.35	90.35
Potassium Phosphate, Monobasic, Anhydrous (KH_2PO_4)	85.0	85.0
Myo-Inositol ($C_6H_{12}O_6$)	50.0	50.0
EDTA, Disodium Salt, Dihydrate ($C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$)	18.63	18.63
Ferrous Sulfate, Heptahydrate ($FeSO_4 \cdot 7H_2O$)	13.9	13.9
Manganese Sulfate, Monohydrate ($MnSO_4 \cdot H_2O$)	8.45	8.45
Zinc Sulfate, Heptahydrate ($ZnSO_4 \cdot 7H_2O$)	4.3	4.3
Boric Acid (H_3BO_3)	3.1	3.1
Glycine ($C_2H_5NO_2$)	1.0	1.0
Potassium Iodide (KI)	0.415	0.415
Nicotinic Acid ($C_6H_5NO_2$)	0.25	0.25
Pyridoxine, Hydrochloride ($C_8H_{11}NO_3 \cdot HCl$)	0.25	0.25
Molybdic Acid Sodium Salt, Dihydrate ($Na_2MoO_4 \cdot 2H_2O$)	0.125	0.125
Thiamine, Hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$)	0.05	0.05
Cobalt Chloride, Hexahydrate ($CoCl_2 \cdot 6H_2O$)	0.0125	0.0125
Cupric Sulfate, Pentahydrate ($CuSO_4 \cdot 5H_2O$)	0.0125	0.0125
Ultrapure water	Up to 1 L	Up to 1 L

Adjust pH to 5.7 by KOH. Filter sterilization by using a 0.22 μm filter. Store at room temperature (23°C–25°C) in the dark for up to 6 months.

1/4 MS inorganic nitrogen-containing or inorganic nitrogen-free medium

Reagent	1/4 MS inorganic nitrogen-containing (mg/L)	1/4 MS inorganic nitrogen-free (mg/L)
Glucose	1800.0	1800.0
MES	1000.0	1000.0
Potassium Nitrate (KNO_3)	475.0	0.0
Ammonium Nitrate (NH_4NO_3)	412.5	0.0
Potassium Sulfate, Anhydrous (K_2SO_4)	0.0	409.4725
Potassium Chloride (KCl)	0.0	177.4775
Calcium Chloride, Anhydrous ($CaCl_2$)	83.05	83.05
Magnesium Sulfate, Anhydrous ($MgSO_4$)	45.175	45.175
Potassium Phosphate, Monobasic, Anhydrous (KH_2PO_4)	42.5	42.5
Myo-Inositol ($C_6H_{12}O_6$)	25.0	25.0
EDTA, Disodium Salt, Dihydrate ($C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$)	9.315	9.315
Ferrous Sulfate, Heptahydrate ($FeSO_4 \cdot 7H_2O$)	6.95	6.95
Manganese Sulfate, Monohydrate ($MnSO_4 \cdot H_2O$)	4.225	4.225
Zinc Sulfate, Heptahydrate ($ZnSO_4 \cdot 7H_2O$)	2.15	2.15
Boric Acid (H_3BO_3)	1.55	1.55
Glycine ($C_2H_5NO_2$)	0.5	0.0
Potassium Iodide (KI)	0.2075	0.2075
Nicotinic Acid ($C_6H_5NO_2$)	0.125	0.125
Pyridoxine, Hydrochloride ($C_8H_{11}NO_3 \cdot HCl$)	0.125	0.125

(*Continued on next page*)

Continued

Reagent	1/4 MS inorganic nitrogen-containing (mg/L)	1/4 MS inorganic nitrogen-free (mg/L)
Molybdic Acid Sodium Salt, Dihydrate ($Na_2MoO_4 \cdot 2H_2O$)	0.0625	0.0625
Thiamine, Hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$)	0.025	0.025
Cobalt Chloride, Hexahydrate ($CoCl_2 \cdot 6H_2O$)	0.00625	0.00625
Cupric Sulfate, Pentahydrate ($CuSO_4 \cdot 5H_2O$)	0.00625	0.00625
Ultrapure water	Up to 1 L	Up to 1 L

Adjust pH to 5.7 by KOH. Filter sterilization by using a 0.22 μm filter. Store at room temperature (23°C–25°C) in the dark for up to 6 months.

Preparation of buffers and solutions

1× PBS buffer pH 7.4

Reagent	Working concentration	Amount for 1 L
KCl	3 mM	0.22 g
KH_2PO_4	1.8 mM	0.24 g
NaCl	137 mM	8.0 g
Na_2HPO_4	10 mM	1.42 g
Ultrapure water	-	To make up 1 L

Adjust pH to 7.4 by HCl. Autoclaved at 121°C, 15 min. The buffer can be stored at room temperature (23°C–25°C) for up to one year.

Fixer buffer

Reagent	Stock concentration	Working concentration	Amount for 10 mL
PBS pH 7.4	2 X	1 X	5.0 mL
Formaldehyde	37–40% (v/v)	4% (v/v)	1–1.081 mL
Triton X-100	10% (v/v)	0.1% (v/v)	0.1 mL
Ultrapure water	-	-	To make up 10 mL

The fixer buffer is recommended to prepare freshly before use.

EdU detection cocktail solution

Reagent	Stock solution/concentration	Amount for 1 sample
Buffer addictive	Component F (store at - 20°C)	1.6 μL
Reaction buffer	Component D (store at 4°C)	14.0 μL
Copper (II) sulfate solution	Component E (100 mM $CuSO_4$)	6.7 μL
Alexa Fluor 488 azide	Component B (store at 4°C)	0.07 μL
Ultrapure water	-	144.0 μL

The Edu detection cocktail solution should be prepared freshly before use.

0.2 M Sodium phosphate buffer pH7.0

Reagent	Stock concentration	Amount for 100 mL
NaH_2PO_4	0.2 M	39.0 mL
Na_2HPO_4	0.2 M	61.0 mL

Adjust pH to 7.0. Autoclaved at 121°C, 15 min. The buffer can be stored at room temperature (23°C–25°C) for up to one year.

GUS staining solution			
Reagent	Stock concentration	Working concentration	Amount for 100 mL
Sodium phosphate buffer pH7.0	0.2 M	50 mM	25.0 mL
$K_3[Fe(CN)_6]$	0.1 M	0.4 mM	0.4 mL
$K_4Fe(CN)_6 \cdot 3H_2O$	0.1 M	0.4 mM	0.4 mL
Triton X-100	10% (v/v)	0.05% (v/v)	0.5 mL
EDTA	0.5 M	8 mM	1.6 mL
Methanol	100% (v/v)	20% (v/v)	20.0 mL
X-Gluc	-	0.8 g/mL	80.0 mg
Ultrapure water	-	-	To make up 100 mL
GUS staining solution (without X-Gluc) could be prepared and stored at 4°C in the dark for up to 6 months. X-Gluc (5-bromo-4-chloro-3-indolyl-b-D-glucuronide) needs to be added freshly before use.			

2× SDS protein extraction buffer			
Reagent	Stock concentration	Working concentration	Amount for 10 mL
Tris-HCl pH 6.8	1 M	80 mM	0.8 mL
β-mercaptoethanol	14.4 M	4 mM	2.8 μL
SDS	20% (v/v)	4% (w/v)	2.0 mL
Glycerol	100% (v/v)	8% (v/v)	0.8 mL
Bromophenol blue	-	0.02% (w/v)	2.0 mg
Ultrapure water	-	-	To make up 10 mL
The buffer can be stored at 4°C for up to one month.			

TBST buffer			
Reagent	Stock concentration	Working concentration	Amount for 1 L
Tris-HCl	1 M	20 mM	20 mL
NaCl	5 M	137 mM	27.4 mL
Tween-20	100% (v/v)	0.05% (v/v)	0.5 mL
Ultrapure water	-	-	To make up 1 L
Adjust pH to 7.6 with NaOH. The buffer can be stored at 4°C for up to one month.			

STEP-BY-STEP METHOD DETAILS

Liquid culture system for glucose-TOR signaling study

Preparation mitotic quiescent roots by sugar depletion

Timing: 5 days

Prepare sugar depletion seedlings with mitotic quiescent roots.

1. Add 1 mL of 1/2 MS sugar-free or sugar-containing (0.5% (w/v)) liquid medium to each well of a 6-well plate.

   Note: The volume of media in the 6-well plate should be 1 mL per well for ideal plant growth.

   Note: 6-well plates are highly recommended for this assay. To fit the experiments that need less or more plant materials, 12-well or 9 cm round plates could be also used. But the volume of media per well should be scaled down or up based on the well size.

2. Sow 10 surface-sterilized WT Arabidopsis seeds in each well.

Note: The number of seeds for each well should be identical for each biological experiment, and no more than 15.

3. Grow the seedlings in an environment-controlled growth room or chamber at 22°C, 65% humidity, and 75 μmol m^{-2} s^{-1} light intensity under a 12 h light/12 h dark photoperiod for up to 4 days after germination (DAG). Troubleshooting 1
4. Shake the 6-well plates gently once by hand every day to help the uniform distribution of nutrients in the liquid medium.
5. Replace the liquid medium in each well by 1 mL of fresh medium at day 4, or before any treatments.

Note: DAG: Day After Germination. In this protocol, 4 DAG refers the 5^{th} day after sowing because *Arabidopsis* seeds need one day to germinate in the medium.

Note: Replacing the liquid medium at day 4 or before the treatments is critical. Replacing of the medium is to ensure that other nutrients are not exhausted during the incubation process. The final volume of medium in each well is exactly one milliliter, and all nutrients excepts for the glucose are the same among the different experimental samples.

⚠ CRITICAL: One milliliter, neither more nor less, of media in each well of 6-well plate is critical for depleting the plant's endogenous sugar in this liquid culture system. Less than one milliliter will not efficiently block the carbon dioxide absorption by the plant seedlings, which may then trigger weak photosynthesis activity in the seedlings and cause insufficient sugar depletion. More than one milliliter medium in the well might cause undesired hypoxia responses.

Phenotype observation and analysis of sugar depletion seedlings

Timing: 2–4 days

Analysis the root growth phenotype in the sugar depletion seedlings

6. Capture the photos of the seedlings growing in sugar-free or sugar-containing medium at 1 to 4 DAG (Figure 1A).

Note: The roots of the sugar depletion seedlings will stop growing at 3 to 4 DAG.

7. Analyze the primary root growth phenotype, e.g., measure the root length by software image J, observe the root apical meristem length or cell number by using a Leica DIC microscopy or examine the cell proliferation activity by EdU staining.
8. Comparison of the root length of seedlings grown in sugar-free or sugar-containing medium to evaluate tnitrogen starvation depletion on root growth (Figure 1B).

EdU staining (by Click-iT EdU alexa Fluor 488 HCS kit) to detect the proliferation activity of the root apical meristem cells

Timing: 4–6 h

Evaluate the cell proliferation activity in root apical meristem of the sugar depletion seedlings

9. Treat the 1 to 4 DAG of WT seedlings grown in sugar-free or sugar-containing medium in a 6-well plate with 1 μM EdU (add 1 μL of 1 mM EdU in each well with 1 mL medium) for 30 min before sample collection.

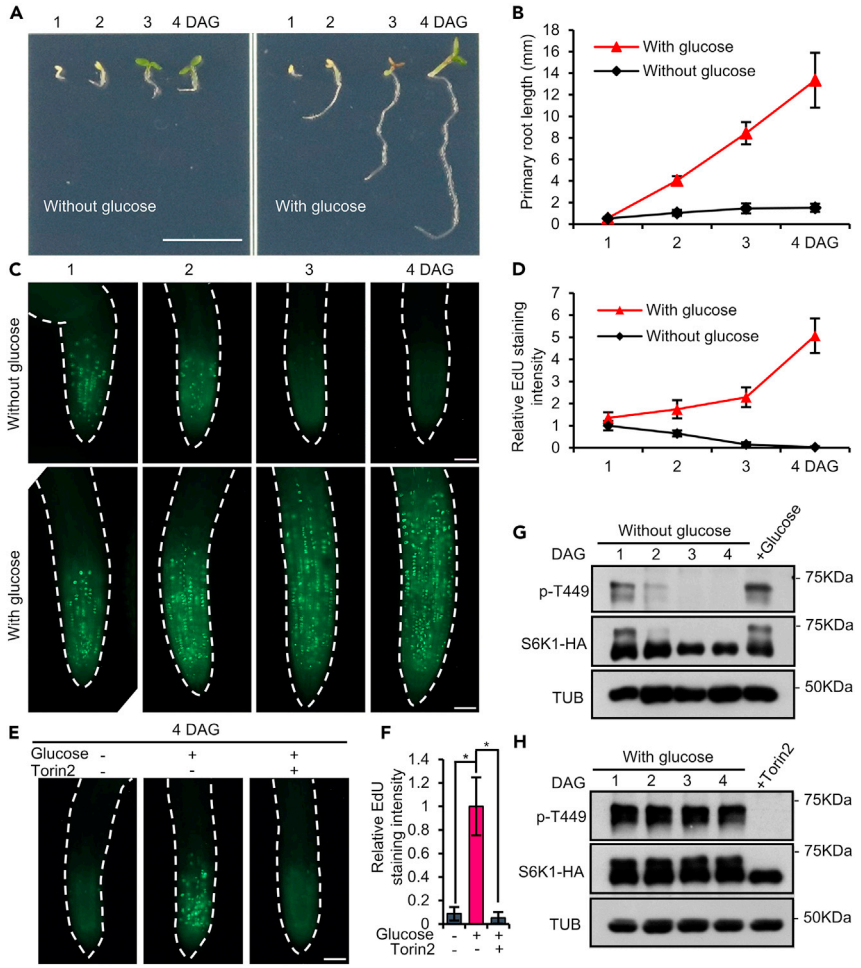

Figure 1. Glucose-TOR signaling regulates root meristem activity

(A) Glucose depletion inhibited *Arabidopsis* primary root growth. DAG, day after germination. Scale bar: 5 mm.

(B) Quantification of primary root length in (A). n ≥10. Data are means ± SD.

(C) Root apical meristem activities, indicated by EdU staining, were gradually decreased by glucose depletion. Scale bar: 50 μm.

(D) Quantification of EdU staining intensity in (C). n ≥10. Data are means ± SD.

(E) Glucose re-activation of the quiescent root apical meristem required TOR kinase. 4 DAG seedlings were recovered for 2 h by 15 mM glucose (0.27% (w/v)) with or without Torin2 (5 μM). Scale bar: 50 μm.

(F) Quantification of EdU staining intensity in (E). n ≥10. Data are means ± SD. *p< 0.05 (two-sided Student's *t* test).

(G) TOR kinase activity in the seedlings were decreased by glucose depletion, and could be quickly reactivated by resupplying 15 mM glucose (0.27% (w/v)) for 15 min *35S::S6K1-HA* seedlings were germinated in sugar-free medium (without glucose) and were treated as indicated.

(H) Seedlings grown in glucose-containing medium for the indicated DAG maintained relatively high and stable TOR kinase activity. 4 DAG of *35S::S6K1-HA* seedlings were treated with Torin2 (5 μM) for 1 h.

Note: In this step, 4 DAG glucose depletion seedlings pretreated with DMSO for 1 h before recovery with 15mM glucose (0.27% (w/v)), a concentration that is sufficient to fully substitute for the photosynthetic support of root meristem activation and the acceleration of root growth (Xiong et al., 2013), for 2 h act as the positive treatment group. 4 DAG glucose depletion seedlings pretreated with 5 μM Torin2 (TOR kinase specific inhibitor) for 1 h before a 2 h glucose recovery is used as the negative control.

Note: Since the chemicals need to be absorbed into the seedlings to inhibit TOR kinase activity, we recommend pretreating with the inhibitors for about an hour rather than adding

inhibitor and glucose together at the same time so as to avoid the possible leakage activation effect.

Note: Rapamycin and several secondary generation mTOR specific inhibitors (e.g. AZD-8055, Torin1 and Torin2), have been successfully used for plant TOR signaling studies (Fu et al., 2021; Li et al., 2017; Liu et al., 2021; Montané and Menand, 2019; Xiong et al., 2013). The optimized treatment time and concentration for a specific inhibitor could be experimentally verified based on the experimental design and purposes (See an example in Extended Data Figure 2C, (Fu et al., 2021)).

10. Collect the seedlings into 1.5 mL Eppendorf tubes.
11. Fix the seedlings with fixer buffer (4% (v/v) formaldehyde solution in 1× PBS (pH 7.4) buffer with 0.1% (v/v) Triton X-100) for 30 min.
12. Discard the fixer and wash the seedlings with 1× PBS buffer 3 times, 10 min each time.

Pause point: This assay can be stopped after performing step 12. The fixed seedlings can be stored at 4°C in 1× PBS buffer for 7 days.

13. Incubate in EdU detection cocktail for 30 min in the dark.
14. Discard the EdU detection cocktail and wash with PBS buffer three times, 10 min each time.
15. Observe the root meristem cells and capture photos with a Leica DM6B fluorescence microscope under the channel with an L5 filter (excitation: around 488 nm). The settings of microscope and imaging are: 1) argon laser intensity (turn to maximum), 2) light mode: fluorescence intensity manager (FIM), 3) FIM intensity: 10%, IL-Fld: turn to full, 4) exposure time: 2.98 s, gain value: 3 (Figures 1C and 1E). Troubleshooting 2

Note: EdU staining is a stable chemical labeling method. It is not necessary to observe the EdU signal right after the last washing step. But we recommend finishing the observation within 12 h.

16. Analyze the root meristem cell proliferation activity by measuring the fluorescent intensity and counting the number of EdU staining dots to evaluate tnitrogen starvation depletion on root meristem activity (Figures 1D and 1F).

Note: More detail information for the EdU staining assay please refer to https://www.thermofisher.cn/order/catalog/product/C10350#/C10350.

Note: Besides EdU staining, other optional methods could also be used to monitor the cell proliferation activity in the root apical meristem, such as using the cell cycle reporter lines *pCYCB1; 1::GUS* (Li et al., 2017), or PlaCCI (Plant Cell Cycle Indica tor) (Desvoyes et al., 2020).

Note: In addition to the root apical meristem, the cell proliferation activity in the shoot apex is also decreased to a very low level in 4 DAG glucose depletion seedlings (Li et al., 2017).

Liquid culture system for Nitrogen-TOR signaling study

Preparation mitotic quiescent shoots by inorganic nitrogen depletion

Timing: 10 days

Prepare inorganic nitrogen depletion seedlings with mitotic quiescent shoots.

17. Add 1 mL of 1/4 MS nitrogen-free or nitrogen-containing liquid medium in each well of 6-well plates.

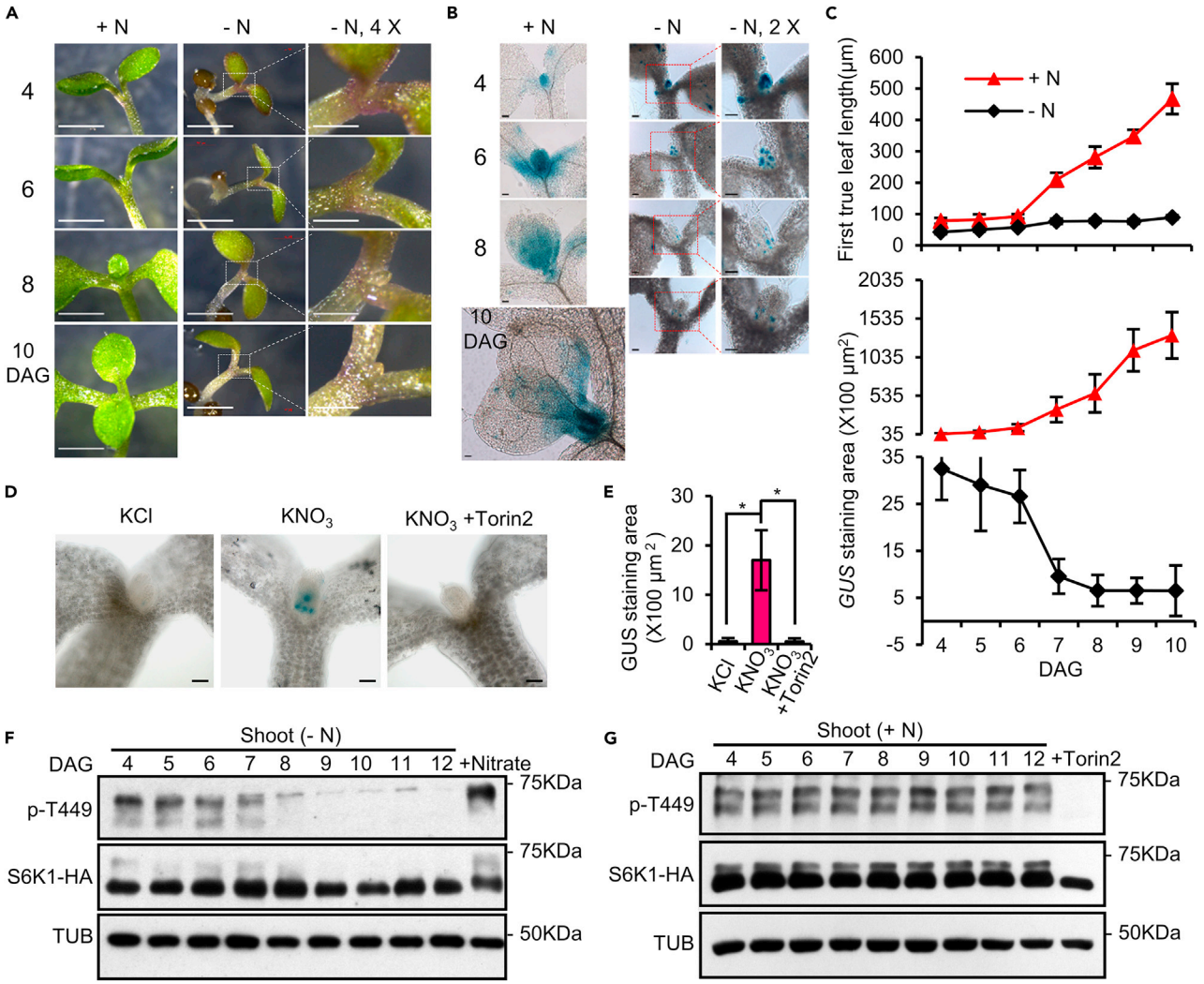

Figure 2. Nitrogen-TOR signaling regulates shoot apexes cell division activity

(A and B) Inorganic nitrogen (N) depletion inhibited *Arabidopsis* true leaf development and cell proliferation in leaf primordium, indicated by GUS staining of *pCYCB1; 1::GUS* seedling. DAG, day after germination. Scale bar: 1 mm (the first and second column of A), 250 μm (the third column in A), 50 μm (B).

(C) Quantification of true leaf size and GUS staining area in (A) and (B); n ≥20. Data are means ± SD.

(D) Nitrate activation of cell proliferation required TOR, indicated by GUS staining of *pCYCB1; 1::GUS* seedlings. Scale bar: 50 μm. *pCYCB1; 1::GUS* seedlings were grown in inorganic nitrogen-free medium for 9 DAG, then treated with 1 mM nitrate (0.01% (w/v)) for 6 h with or without Torin2 (pretreat for 1 h before nitrate recovery, 5 μM).

(E) Quantification of GUS staining area in (D); n ≥15. Data are means ± SD; *p < 0.05 (two-sided Student's t test).

(F) TOR activity in shoot apices gradually decreased in response to inorganic nitrogen depletion (- N) and the decreased TOR activity could be quickly recovered by resupplying 1 mM nitrate (0.01% (w/v)) for 30 min in 9 DAG inorganic nitrogen depletion seedlings. *35S::S6K1-HA* seedlings were germinated in inorganic nitrogen-free medium and were treated as indicated.

(G) Seedlings grown in inorganic nitrogen-containing medium (+ N) for 4–12 DAG maintained relatively high and stable TOR kinase activity. 9 DAG of *35S::S6K1-HA* seedlings were treated with 5 μM Torin2 for 1 h.

Figure reprinted with permission from Liu et al. (2021).

Note: 6-well plate is highly recommended for this assay. To fit the experiments that need less or more plant materials, 12-well or 9 cm round plates could also be used. But the volume of media per well should to be scaled up or down based on the size of the wells.

18. Plant 10 surface-sterilized WT or *pCYCB1; 1::GUS Arabidopsis* seeds in each well.

Note: The number of seeds for each well should be identical for each biological experiment and not exceed 15 seeds.

19. Grow the plants in an environment-controlled growth room or chamber at 22°C, 65% humidity, and 75 μmol m^{-2} s^{-1} light intensity under a 12 h light/12 h dark photoperiod for up to 10 DAG. Troubleshooting 3
20. Shake the 6-well plates gently once by hand every day to help the uniform distribution of nutrients in the liquid medium.
21. Refresh the growth medium every 4 days during the incubation progress and refresh the medium before recovery or treatment assays.

Note: The main purpose of refreshing the growth medium every 4 days is to ensure that glucose and other nutrients are not exhausted during this incubation process. Refreshing the medium before treatment is to make sure the final volume of growth medium in each well is exactly one milliliter, and all macro- and micro-nutrients excepts for nitrogen are the same among different experimental samples .

Phenotype observation and analysis of nitrogen depletion seedlings

Timing: 10 days

Analysis of shoot growth phenotype in the inorganic nitrogen depletion seedlings

22. Observe the shoot phenotype every day and capture the photos of the seedlings growing in nitrogen-free or nitrogen-containing medium at 4 to 10 DAG under a Leica stereoscope microscope (Figure 2A). Measure the first true leaf length by software image J.

Note: The true leaf of seedlings grown in nitrogen-free medium will cease growing at 8 to 10 DAG.

23. Analyze the first true leaf length of seedlings to evaluate the effect of nitrogen depletion on shoot growth (Figure 2C).

GUS staining to detect the cell proliferation activity in the leaf primordium

Timing: 2 days

Evaluate the proliferation activity in the leaf primordium by detecting the *pCYCB1; 1::GUS* expression level in shoot apexes of the inorganic nitrogen depletion seedlings.

24. Dip the 4–10 DAG of *pCYCB1; 1::GUS* seedlings grown in nitrogen-free or nitrogen-containing medium in 3 mL GUS staining solution, and incubate at 37°C for 12 h in the dark.

Note: In this step, 9 DAG nitrogen depletion seedlings are pretreated with DMSO for 1 h before recovery with 1 mM KNO_3 (0.01% (w/v)) for 6 h. This is the set up for the positive control. 9 DAG nitrogen depletion seedlings pretreated with DMSO for 1 h before recovery with 1 mM KCl (0.0075% (w/v)), or pretreated with 5 μM Torin2 for 1 h before recovery with 1 mM KNO_3 (0.01% (w/v)), respectively, are used as the negative controls.

Note: For the quantification of GUS staining intensity, GUS staining time is important in this step. A shorter time may cause insufficient staining, longer time may lead to unspecific staining.

25. De-stain the seedlings with 75% (v/v) ethanol at room temperature (23°C–25°C), add 3 mL of 75% (v/v) ethanol in each well of 6-well plate and replace the ethanol every hour until the leaves of seedlings turn transparent and white.
26. Observe the GUS staining in the shoot apexes and capture the photos using a Leica microscope (Figures 2B and 2D). Troubleshooting 4

Note: The cell division activity in leaf primordium is continually decreased from 4 to 10 DAG.

Note: In the sugar depletion system described above, both the root apical meristem activity and shoot apex cell division activity are decreased to a very low level after 4 DAG. However, for the inorganic nitrogen depletion system, only the shoot apex cell division activity is decreased to a very low level after 9 DAG. The root apical meristem cell division activity still remains at a relatively higher level, which might be caused by nitrogen nutrient remobilization between the shoot and root parts (For details, see Figures 1A–1D, (Liu et al., 2021)).

27. Analyze the GUS staining area in shoot apexes by software image J to evaluate the effect of nitrogen depletion on cell proliferation activity in the shoot apex (Figures 2C and 2E).

Protein blots analyses of TOR activity based on P-T449 level of S6K1

Timing: 1–2 days

To investigate tnitrogen starvation /nitrogen starvation on endogenous TOR kinase activity, we monitored TOR kinase activity based on the phosphorylation of T449 in the TOR-conserved substrate ribosomal S6 kinase 1 (S6K1, (Xiong and Sheen, 2012); Figures 1G, 1H, 2F, and 2G).

28. Prepare sugar/ nitrogen depletion and sugar/ nitrogen recovery samples of *35S::S6K1-HA* as described above.

Note: 10 mg of plant powder is enough for 3 rounds of protein blots analyses. The collected samples can be stored at −80°C for up to 30 days.

Note: The *35S::S6K1-HA* transgenic line (in Col-0 background) shows no obvious phenotype compared with Col-0, and has been used to monitor the *in vivo* TOR kinase activity in multiple studies (Cai et al., 2017; Fu et al., 2021; Li et al., 2017; Liu et al., 2021; O'Leary et al., 2020; Xiong et al., 2013; Xiong and Sheen, 2012; Zhang et al., 2019).

Note: In this step, positive controls, e.g. sugar/nitrogen recovery samples, and negative controls, e.g. 5 μM Torin2 (TOR kinase specific inhibitor) treatment samples are strongly recommended.

Note: In addition to S6K1-pT449, as an alternative method, RPS6-pS240, a direct phosphorylation site of S6K1, was also used to monitor the endogenous TOR kinase activity in recent years (Dobrenel et al., 2016).

29. Grind the samples into fine powder with a mortar and pestle in liquid nitrogen.
30. Add 60 μL of 2× SDS protein extraction buffer in 10 mg plant powder and vortex for 15 s.
31. Boil the protein extracts at 95°C for 5 min.
32. Spin at 13523 X g for 2 min at 4°C.
33. Take 20 μL of the supernatants and load onto an 8% SDS-PAGE gel. Run with constant voltage at 80 V for 15 min, then turn to constant voltage at 150 V for 40 min. The SDS-PAGE gel running is

performed at room temperature (23°C–25°C). Transfer proteins to a PVDF membrane using standard western blot procedures.

34. Block the membrane with 5% (w/v) nonfat milk dissolving in TBST buffer for 1 h at 25°C with shaking.
35. Wash the membrane with TBST buffer 3 times with agitation (10 min each time), and incubate the membrane with the primary antibodies (S6K1 phosphorylation antibody P-T449, 1:2000 in 5% (w/v) BSA in TBST buffer; conjugated-HRP HA antibody, 1: 5000 in 5% (w/v) milk in TBST buffer; Tubulin antibody, 1:5000 in 5% (w/v) milk in TBST buffer) at 4°C for 12 h (overnight) with agitation.
36. Wash the membrane with TBST buffer 3 times with agitation (10 min each time), and incubate with the secondary antibodies in 5% (w/v) milk in TBST buffer (for S6K1-P-T449, with goat anti-rabbit antibody, 1:5000; for Tubulin, with rabbit anti-mouse antibody) for 1 h at 25°C with shaking.

Note: The antibodies used in step 35 and 36 can be reused 10 to 15 times. The anti-HA antibody we used in step 35 is a conjugated-HRP anti-HA antibody. So it does not need to incubate with the secondary antibody before incubation with chemiluminescent subatrate.

37. Wash the membrane with TBST buffer 3 times with agitation (10 min each time), and incubate the blot with 2 mL SuperSignal West Pico PLUS chemiluminescent substrate for 5 min in the dark.
38. Expose to film for 30 s to 1 min and develop the film in an ECOMAX X-Ray film processor with developing solution and fixing solution in the dark room. Troubleshooting 5

EXPECTED OUTCOMES

Seedlings grown in 1 mL sugar-free 1/2 MS liquid medium exhibit short roots and absence of true leaves, which are caused by low cell division activity in root meristem (monitored by EdU staining, Figures 1A–1D) and in leaf primordium (Li et al., 2017) during the sugar depletion progress. This system will generate mitotic quiescent root and shoot apices at 3–4 DAG. Furthermore, the decrease of cell division activity in the root and shoot apex caused by sugar depletion highly rely on the activity of the central nutrient signaling regulator TOR kinase (Figures 1E–1H, and (Li et al., 2017)).

Seedlings grown in 1 mL inorganic nitrogen-free 1/4 MS liquid medium show shoot apex growth arrest, which is caused by a decrease of cell division activity in the shoot apexes (monitored by *pCYCB1; 1::GUS* expression) during inorganic nitrogen depletion progress (Figures 2A–2C). This system will generate mitotic quiescent shoot apexes by inorganic nitrogen depletion at 8–9 DAG (Figures 2A–2C). Furthermore, the decrease of cell division activity in shoot apexes caused by inorganic nitrogen depletion highly rely on the activity of TOR kinase (Figures 2D–2G).

Therefore, this liquid culture system provides an ideal system for investigating nutrient signaling in plant cell proliferation, e.g., sugar-TOR and nitrogen-TOR signaling in plant cell proliferation regulation.

QUANTIFICATION AND STATISTICAL ANALYSIS

The primary root length, first true leaf length, EdU fluorescence intensity, and GUS staining area were measured and quantified with the software Image J (v1.8.0) (NIH; https://imagej.nih.gov/ij/). Statistical significance analysis of the above data were examined by two-sided student's *t* test (* $p < 0.05$).

LIMITATIONS

The current protocol for studying sugar/nitrogen-TOR signaling is limited by exclusively using plants at the seedling stage. The age of seedlings for these assays is usually less than one week, and should not be exceed 15 days. Seedlings older than 15 days are too large to maintain in 6-well plates, and

the small volume of nutrient media (1 mL) may cause unexpected nutrient limitations or other stresses for older plants.

TROUBLESHOOTING

Problem 1

The root growth of seedling in sugar-free liquid medium is not stopped at 3 to 4 DAG (step 3).

Potential solution

There are two reasons that might lead to the continuously root growth. One is that the medium may be contaminated by sugar, and the other one is that the volume of liquid medium in each well is less than 1 mL, which will cause weak photosynthesis activity. The potential solution is to re-prepare the sterilized 1/2 MS sugar- free medium or re-fresh the liquid medium to make sure the volume is exactly 1 mL.

Problem 2

The EdU staining background signal in the root apical meristem is too high (step 15).

Potential solution

There are two ways to decrease the background signal of EdU staining. One is to increase the wash buffer volume or the times of washing in step 12 and 14, and the other one is to adjust the exposure time or laser intensity of the microscope to a suitable observation setting.

Problem 3

The root growth of seedling in nitrogen-free liquid medium is arrested early (step 19).

Potential solution

The root of seedlings in inorganic nitrogen-free medium could continuously grow until 18–20 DAG. There are two reasons that might lead to the early arrest of root growth. One is that the quality of seeds is bad, the other one is that the medium may be contaminated by fungi or bacteria. The potential solution is to re-prepare the seedlings with high quality seeds and to prepare new sterilized 1/4 MS nitrogen- free medium.

Problem 4

The background of inorganic nitrogen-depletion seedlings' leaves of GUS staining are deeper than the leaves of nitrogen-containing seedlings (step 26).

Potential solution

This phenomenon is caused by inorganic nitrogen-depletion treatment, not by GUS staining or the de-staining process. This problem could be alleviated by using the transparent agent (85–90% (v/v) lactic acid). Add 50 μL of lactic acid on the glass slide when loading the seedling on it, wait for 2–3 min, before observation.

Problem 5

The protein blot result of the phosphor-antibody of p-T449 shows non-specific background (step 38).

Potential solution

This could be solved by reusing the phosphor-antibody of p-T449 for 4–5 times to decrease the non-specific background or by increasing the times of washing in step 35 and 37.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Yan Xiong (yanxiong@fafu.edu.cn).

Materials availability

This study did not generate new unique reagents. The plasmids and plant materials generated in this study are available upon request from the Lead Contact.

Data and code availability

This study did not generate new codes. Original data have been deposited to Mendeley Data: https://dx.doi.org/10.17632/d2d9tpjtv4.1 and https://doi.org/10.17632/h8px3kzd6g.3.

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AUTHOR CONTRIBUTIONS

Conceptualization, Y.L. and Y.X.; Investigation, Y.L.; Writing – Original Draft, Y.L.; Writing – Review & Editing, Y.X.; Funding Acquisition, Y.L. and Y.X.; Supervision, Y.X.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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