

Imaging of Chloroplast Movement Responses to Light Stimulation in Different Intensities in Rice

Hui Wu[#], Ziyi Wang[#], Yanchun Yu^{*}, and Yanli Zhang^{*}

College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou 310036, China

*For correspondence: ycyu@hznu.edu.cn; zhangyanli1121@sina.com

[#]Contributed equally to this work

Abstract

Chloroplast movement has been observed and analyzed since the 19th century. Subsequently, the phenomenon is widely observed in various plant species such as fern, moss, *Marchantia polymorpha*, and *Arabidopsis*. However, chloroplast movement in rice is less investigated, presumably due to the thick wax layer on its leaf surface, which reduces light sensitivity to the point that it was previously believed that there was no light-induced movement in rice. In this study, we present a convenient protocol suitable for observing chloroplast movement in rice only by optical microscopy without using special equipment. It will allow researchers to explore other signaling components involved in chloroplast movement in rice.

Keywords: Imaging, Chloroplast movement, Rice, Optical microscope, Light

This protocol was validated in: Plant Physiol (2022), DOI: 10.1093/plphys/kiac427

Background

The chloroplast, as a plant-specific photosynthetic organelle, has different responses to different light intensities (Kong and Wada, 2016). Under weak light, chloroplasts accumulate at the front face of the cells to absorb enough light for photosynthesis; under strong light, chloroplasts move to the anticlinal cell wall to avoid photo-damage (Wada, 2013). In model systems such as *Arabidopsis thaliana*, a number of components and downstream signaling proteins have been shown to mediate chloroplasts movement signaling in response to different light conditions (Kong and Wada, 2014). However, the molecular mechanism of chloroplast movement in rice still remains unclear, due to limitations on experimental methods and detection tools.

The main methods used for detecting chloroplast movement include: (i) green and white band assay, (ii) optical microscope, (iii) measurement of red-light transmittance, (iv) confocal laser scanning microscope, and (v) leaf cross section. Due to the complexity of rice's leaf structure, the existing methods are not suitable (Suetsugu et al., 2017). In this protocol, we describe a method to detect chloroplast movement using rice leaf sheath. The method is based on a protocol established for *Arabidopsis* and rice (Zhang et al., 2022), which we have adapted and optimized for convenient and accurate analysis of rice and other gramineous plants. There is no doubt that the system will be beneficial for further understanding of the molecular mechanisms and evolutionary history of chloroplast movement in green plants.

Materials and Reagents

1. 9 cm Petri dish (Sangon Biotech, catalog number: F611001)
2. Hydroponic tank (Koraba, catalog number: 53506733434)
3. Razor blade and forceps
4. 1.5 mL tube (Nantong Haidi Experimental Equipment Co., Ltd, catalog number: B030003)
5. Sealing film (Parafilm, catalog number: pm996)
6. Microscope slides, 76 × 26 mm (Sangon Biotech, catalog number: F518101)
7. Cover slides, 24 × 60 mm (Sangon Biotech, catalog number: F518118)
8. Rice seeds (Shuhui 527, *indica*)
9. Glycerol (Sangon Biotech Co., Ltd, catalog number: A501745-0500)
10. Mounting medium (Thermo Fisher, catalog number: 008030)
11. NH₄Cl (Sigma, catalog number: A9434-500G)
12. Ca(NO₃)₂·4H₂O (Sigma, catalog number: C1396-500G)
13. MgSO₄·7H₂O (Sigma, catalog number: M2773-500G)
14. K₂SO₄ (Sigma, catalog number: P0772-250G)
15. NaH₂PO₄·2H₂O (Sigma, catalog number: 71500-250G)
16. FeSO₄·7H₂O (Sigma, catalog number: F7002-500G)
17. Na₂EDTA·2H₂O (Sigma, catalog number: 03685-500G)
18. H₃BO₃ (Sigma, catalog number: B0394-100G)
19. ZnSO₄·7H₂O (Sigma, catalog number: Z0251-500G)
20. CuSO₄·5H₂O (Sigma, catalog number: 209198-250G)
21. MnCl₂·4H₂O (Sigma, catalog number: M3634-100G)
22. (NH₄)₆Mo₇O₂₄·4H₂O (Sangon Biotech, catalog number: A600067-0100)
23. Formaldehyde (Sigma-Aldrich, catalog number: F8775)
24. Acetic acid (Sigma-Aldrich, catalog number: A6283)
25. Na₂HPO₄·12H₂O (Sangon Biotech, catalog number: A607793)
26. Ethanol (any manufacturer)
27. Culture solution (see Recipes)
28. FAA stationary fluid (see Recipes)
29. 0.2 M PBS (pH 7.0) (see Recipes)

Equipment

1. Constant-temperature incubator (Shanghai Jing Hong Laboratory Instrument Co. Ltd., catalog number: GNP-9080)
2. LED illumination (Fujian Jiupo Biotechnology Co. Ltd., catalog number: WL50)
3. Spectrum analyzer (EVERFINE, catalog number: PLA-30)
4. Eppendorf Research® plus Pipette (100–1,000 µL pipette, catalog number: 3120000062)
5. Vacuum (LABCONCO, CentriVap DNA vacuum concentrator, catalog number: 7970037)
6. Optical microscope (Nikon, model: ECLIPSE Ni-U)

Software

1. ImageJ (National Institutes of Health, <https://imagej.nih.gov/ij/index.html>)

Procedure

A. Rice cultivation

1. Select plump rice seeds, put them in the Petri dish without sterilization, and add 20 mL of distilled water to submerge the seeds. Put the Petri dish into the constant-temperature incubator at 37 °C for two days. During this time, change the water every 12 h (Figure 1A).
2. Select the germinated seeds with an identical radical length. Put them into the wells of the hydroponic tank one by one with the embryo upward. Fill the hydroponic tank with tap water; the liquid level should reach the seed radicle. Put the hydroponic tank in a growth chamber under white light at approximately 50 µmol m⁻² s⁻¹ in a 14:10 h dark/light cycle at 30 °C (Figure 1A).
3. After approximately two days of growth, change the water to culture solution and grow for ten additional days. During growth, replenish with some culture solution when the liquid level gets too low (Figure 1B).

B. Light treatment

1. Turn on the light set at 100%. Use the spectrum analyzer to detect light intensity at different distances from the light plate and find the positions of 50 and 300 µmol m⁻² s⁻¹.
2. Prepare three Petri dishes and fill them with culture solution. Select nine healthy seedlings (three for each Petri dish) and place them horizontally in the Petri dish in parallel. Cover them with a big box for 3 h (Figure 1C).
3. After dark treatment for 3 h, take out two of the Petri dishes and place them at the position where the light intensity is at 50 µmol m⁻² s⁻¹ (weak light) and 300 µmol m⁻² s⁻¹ (strong light); the remaining one remains in the dark (Figure 1D).
4. Keep the seedlings under the light treatment for 2 h.

C. Sampling

1. Prepare three 1.5 mL tubes and add 1 mL of FAA fixative into each tube with a pipette. Mark them as dark, weak light, and strong light. Make a small hole over each tube with a sharp forceps.
2. After the light treatment, cut out small squares (2 × 2 mm, two for each) on the upper side of the leaf sheath with a razor blade and put them into the corresponding tubes (Figure 1E). Samples must be cut in the same environment as the light treatment. Specifically, samples treated with 50 µmol m⁻² s⁻¹ weak light must be cut under 50 µmol m⁻² s⁻¹ weak light; samples treated with 300 µmol m⁻² s⁻¹ strong light must be cut under

300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ strong light. Such operation ensures that the distribution of chloroplasts in the sample will not be changed during sample cutting. Take dark-treated samples under darkness or dim light conditions. Cut a small triangle at the upper-right corner of each sample as a mark to prevent the front and back of the sample from being unclear during observation (Figure 1F).

3. Vacuum the FAA tubes with a vacuum equipment until no air bubble comes out and exhaust slowly to let the fix solution go into the tissue (keeping the tubes under -0.1 MPa for 5 min is enough). Replenish some FAA fixative into the tube if there is any loss in the vacuum process (Figure 1G).
4. Seal the tubes' orifice with sealing film and fix in FAA fixative for 24 h at 4 °C.

D. Observation

1. Wash the samples with 0.2 M PBS three times, each time for 5 min.
2. Place the sample face-up on the slide with a drop of 50% glycerol and cover it gently with a cover slide. Seal the cover slide with mounting medium.
3. Observe the samples on an optical microscope with 100× oil lens and take photos (Figure 1H).

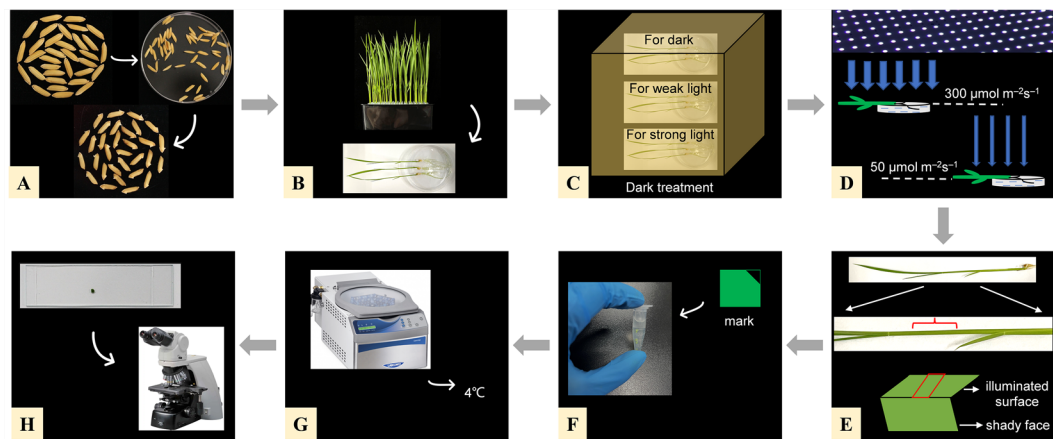


Figure 1. Illustration of the experimental procedure. (A) Seed germination. (B) Cultivate the seedlings for 10 days, select healthy seedlings, and place them in the Petri dishes. (C) Dark treatment for 3 h. (D) Light treatment for 2 h. Take out two of the Petri dishes and place them at the position where the light intensity is 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (weak light) and 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (strong light). (E) Cut out small squares (2 × 2 mm) on the illuminated side of the leaf sheath with a razor blade. (F) Cut a small triangle at the upper right corner of each sample and put them into the FAA fixative. (G) Vacuum the FAA tubes with a vacuum equipment and fix in FAA fixative for 24 h at 4 °C. (H) Observe the samples on an optical microscope with 100× oil lens and take photos.

Data analysis

As shown in Figure 2, different distribution patterns of chloroplasts in wild-type plant cells were visualized. In cells of dark-adapted plants, most chloroplasts were positioned at the bottoms of cells. In the weak white light treatment, most chloroplasts moved to the upper side of the cells. However, the chloroplasts moved along the anticlinal cell walls under strong white light stimulus.

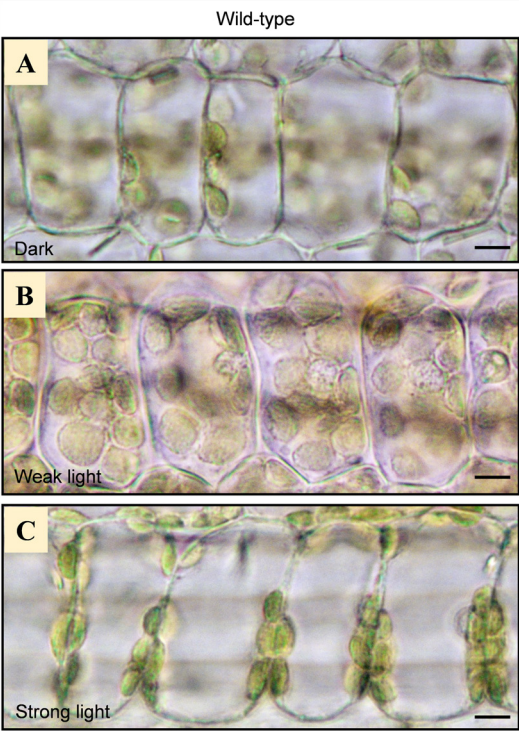


Figure 2. Upper view of wild-type cells. (A) Upper view of wild-type cells under dark. (B) Upper view of wild-type cells under weak light. (C) Upper view of wild-type cells under strong light. Scale bar = 5 μ m.

Recipes

1. Culture solution

For 10 L working culture solution, add 10 mL of nutrient solution I, II, III, IV, and VI, 50 mL of nutrient solution V, and 9.9 L of H₂O. Adjust the pH value to 5.8–6.0 with HCl before use.

Component	Reagent	Amount (g/L)
Nutrient solution I	NH ₄ Cl	58
	Ca(NO ₃) ₂ ·4H ₂ O	142
Nutrient solution II	MgSO ₄ ·7H ₂ O	324
Nutrient solution III	K ₂ SO ₄	71.4
Nutrient solution IV	NaH ₂ PO ₄ ·2H ₂ O	40.3
Nutrient solution V	FeSO ₄ ·7H ₂ O	6.965
	Na ₂ EDTA·2H ₂ O	9.315
Nutrient solution VI	H ₃ BO ₃	0.934
	ZnSO ₄ ·7H ₂ O	0.035
	CuSO ₄ ·5H ₂ O	0.031
	MnCl ₂ ·4H ₂ O	1.5
	(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	0.074

2. FAA stationary fluid

Reagent	Stock concentration	Amount per 100 mL	Final concentration
Formaldehyde	38%	5 mL	1.9%
Acetic acid	100%	5 mL	5%
Ethanol	70%	90 mL	63%
Glycerol	100%	5 mL	5%

3. 0.2 M PBS (pH 7.0)

Solution A: 0.2 M Na_2HPO_4 , 7.164 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ in 100 mL distilled water

Solution B: 0.2 M NaH_2PO_4 , 3.121 g of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ in 100 mL distilled water

Put 61 mL of solution A and 39 mL of solution B together and mix well to make 0.2 M PBS at pH 7.0.

HCl solution is used for adjusting the pH value.

Acknowledgments

This study was supported by Funds from the Natural Science Foundation of Zhejiang (LY20C130004, LGN19C130006) and the National Natural Science Foundation of China (91335103). This protocol was modified from Zhang et al. (2022).

Competing interests

The authors declare no competing interest.

References

- Kong, S. G. and Wada, M. (2014). [Recent advances in understanding the molecular mechanism of chloroplast photorelocation movement](#). *Biochim Biophys Acta* 1837(4): 522-530.
- Kong, S. G. and Wada, M. (2016). [Molecular basis of chloroplast photorelocation movement](#). *J Plant Res* 129(2): 159-166.
- Suetsugu, N., Higa, T. and Wada, M. (2017). [Ferns, mosses and liverworts as model systems for light-mediated chloroplast movements](#). *Plant Cell Environ* 40(11): 2447-2456.
- Wada, M. (2013). [Chloroplast movement](#). *Plant Science* 210: 177-182.
- Zhang, Y., Dong, G., Wu, L., Wang, X., Chen, F., Xiong, E., Xiong, G., Zhou, Y., Kong, Z., Fu, Y., et al. (2022). [Formin protein DRT1 affects gross morphology and chloroplast relocation in rice](#). *Plant Physiol.* doi: 10.1093/plphys/kiac427.