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Rice Lamina Joint Inclination Assay

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[Abstract] Brassinosteroids (BRs) promote rice lamina inclination. Recently, we showed that *OsBUL1* knockout mutant rice (*osbul1*) is defective in brassinosteroid signaling (Jang *et al*., 2017). To show that lamina joint inclination of *osbul1* is less-sensitive than WT to exogenous brassinolide (BL) treatment in the lamina joint inclination bioassays, we applied the protocol presented below. The protocol focuses on: (1) how to prepare rice samples for the assay, and (2) how to treat BL exogenously. Finally, we have added a result showing lamina inclination between WT and *osbul1* in BL solutions of various concentrations.

Keywords: Bioassay, Brassinosteroid, Lamina inclination, Lamina joint, Rice

[Background] The rice lamina joint connects the leaf blade and sheath, contributing significantly to the leaf angle trait and BR is the main regulator of the trait, while other plant hormones, including ethylene, gibberellin, and auxin, also influence leaf angle (Gan *et al*., 2015). A more erect leaf facilitates the penetration of sunlight, enhancing photosynthetic efficiency and occupying less space in dense planting (Sakamoto *et al*., 2006). Thus, rice lamina inclination is one of the major agronomic traits affecting rice plant architecture. Actually, the rice lamina inclination assay developed mainly by Wada and his co-workers is a highly specific and sensitive bioassay for BRs (Wada *et al*., 1981 and 1984). In this bioassay, treatment with BRs induces greater cell expansion of adaxial cells relative to the abaxial cells in the joint regions, causing laminar inclination in a concentration-dependent manner (Takeno and Pharis, 1982; Cao and Chen, 1995). Changes in cell wall extensibility or loosening are essential for cell expansion (Campbell and Braam, 1999). Although the molecular mechanism for such action remains elusive, cell wall loosening enzymes including xyloglucan endotransglycosylase have been shown to be upregulated by BL and involved in this modification, resulting in laminar inclination in rice (Uozu *et al*., 2000). Thus, here we describe a procedure through which we could distinguish the BR sensitivity between the wild type and erect leafed *osbul1* mutant plants through the rice lamina inclination assay.

Materials and Reagents

1. 250 µl pipette tips (Mettler-Toledo International, Rainin, catalog number: 17007479)
2. 1 ml pipette tips (Mettler-Toledo International, Rainin, catalog number: 17001121)

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3. 50 ml SuperClear centrifuge tube (Labcon, catalog number: LAB3181)
4. Filter paper (Advantec, No.1: 90 mm)
5. Petri dish, round, 90 x 15 mm (Alpha Plus, catalog number: 16001-1)
6. 50 ml syringe (Sigma-Aldrich, catalog number: Z124990)
7. Syringe filter (VWR, catalog number: 89041-306)
8. Micropore tape (3M, catalog number: 1530-0)
9. 1 ml tubes
10. Rice seeds: *Orysa sativa* spp. *japonica* cv. Hwayoung and *OsBUL1* knockout mutant rice (*osbul1*)
11. Ethanol (Avantor Performance Materials, J.T. Baker®, catalog number: 8006)
12. Sodium hypochlorite (NaOCl, Commercial Bleach–CLOROX)
13. Tween 20 (Alfa Aesar, Affymetrix/USB, catalog number: J20605)
14. Potassium hydroxide (KOH) (SHOWA, catalog number: 1637-0150)
15. Murashige & Skoog basal medium with Vitamins (MS) (PhytoTechnology Laboratories, catalog number: M519)
16. Sucrose (Alfa Aesar, Affymetrix/USB, catalog number: J21938)
17. Phytogel (Sigma-Aldrich, catalog number: P8169-500G)
18. Brassinolide (BL) (Sigma-Aldrich, catalog number: E1641)
19. Sodium hypochlorite solution (with final available chlorine of 2%) (see Recipes)
20. 5 N potassium hydroxide (KOH) solution (see Recipes)
21. Murashige & Skoog (MS) media (see Recipes)
22. 1 mM Brassinolide (BL) stock solution (see Recipes)

Equipment

1. Rice husker (KETT ELECTRIC LABORATORY, model: TR-130)
2. Ultrasonic cleaner (Elma, model: E-30H)
3. Clean bench (Chu-An, model: MBH-420N)
4. Scissors (Basic Life, catalog number: 76000)
5. Forceps (Basic Life, catalog number: BL6502)
6. Growth chamber (CHANG KUANG, model: CK-68EX)
7. Digital camera (Sony, model: NEX-3N)
8. Protractor (Taiwan united stationery, catalog number: HA401)
9. 600 ml beaker (DWK Life Sciences, DURAN, catalog number: 21 106 48)
10. Glass petri dish (Sun Chion, catalog number: B16A1-0090)
11. Autoclave
12. 10 ml measuring cylinders (DWK Life Sciences, DURAN, catalog number: 21 390 08 04)
13. 100 ml measuring cylinders (DWK Life Sciences, DURAN, catalog number: 21 390 24 02)
14. 500 ml measuring cylinders (DWK Life Sciences, DURAN, catalog number: 21 390 44 03)

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15. Vortex mixer (Vortex-Genie2, Scientific Industries, model: Model G560)
16. Incubator (YIHDER TECHNOLOGY, model: LM-570RD)
17. Pipetmans (Gilson, models: P20, P200 and P1000)
18. RiOs™ Essential 16 Water Purification System (EMD Millipore, model: RiOs™ Essential 16)
19. Summit Series Analytical Balance (Denver Instrument, model: SI-234)
20. pH meter (UltraBasic Benchtop pH Meter, Denver Instrument, model: UB-10)

Software

1. ImageJ (https://imagej.nih.gov/ij/) for lamina angle measurement

Procedure

A. Seedling preparation for lamina inclination
   1. Surface sterilize rice seeds
      a. Remove the lemma and palea of seeds using a rice husker (Figure 1).

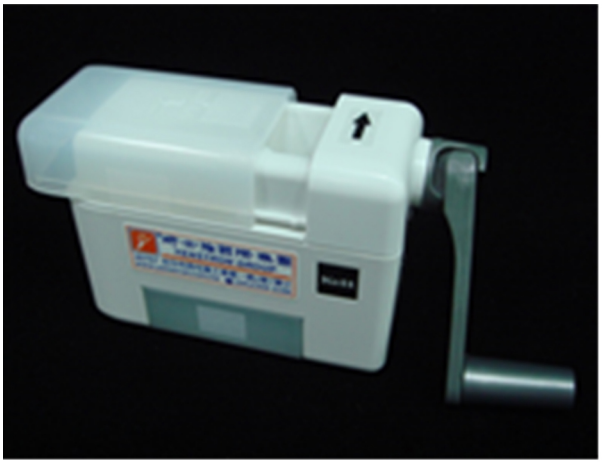

Figure 1. Rice husker used

      b. Put 50-60 naked seeds into a 50 ml SuperClear centrifuge tube and sterilize the surface of the seeds with 30 ml of 70% ethanol for 1 min and vigorously shake by hand.
      c. Rinse the seeds with 30 ml sterile water and pour off the dirty liquid.
      d. Add 30 ml of 2% sodium hypochlorite solution (see Recipes) and place the tube in an ultrasonic cleaner for 20 min.
      e. Discard the sodium hypochlorite solution and wash the seeds with 30 ml sterile water in the clean bench. We usually repeat the washing process 10-15 times.
      f. Place the seeds on autoclaved filter paper to dry and then transfer 20 seeds into a beaker containing MS media (see Recipes) using sterile forceps in the clean bench.
   2. Germinate the sterilized seeds inside a growth chamber under long days (16 h light) at 28 °C

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for 8 days.

3. Sample uniform seedlings (based on similar height in each genotype) by excising approximately 2 cm segments (Figure 2) containing the second-leaf lamina joint, leaf blade and leaf sheath and float excised samples on sterile water for 10 min before transferring them to BL solution.

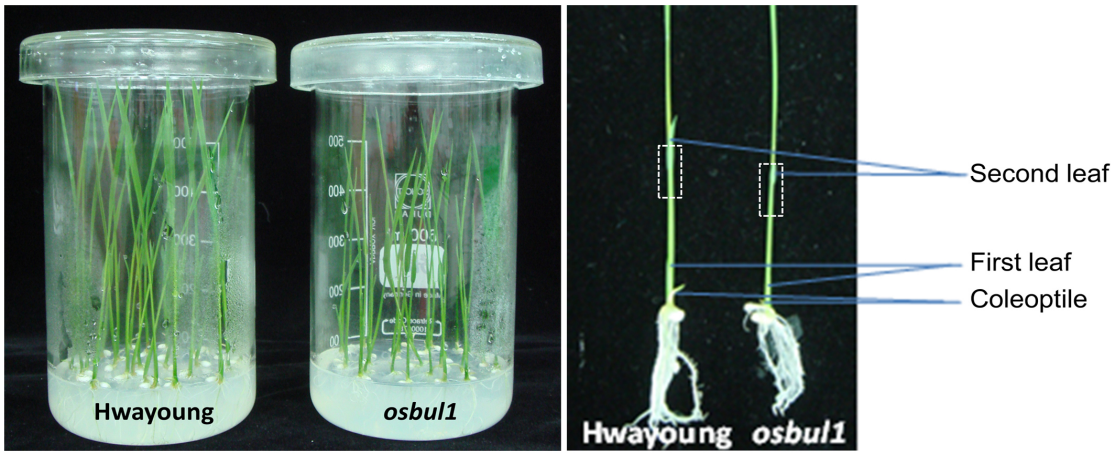

Figure 2. Eight-day-old rice seedlings for lamina inclination assay. WT (Hwayoung cultivar) and *osbul1* mutant rice were grown in beakers containing MS media (left). Leaf segments containing the second-leaf lamina joint of rice seedlings are marked by boxes outlined with dotted lines, which were used for lamina inclination assay.

B. Exogenous BL treatment

1. Put 20 ml of each test BL solution at designated concentrations (0 M, 10^{-6} M, 10^{-7} M, 10^{-8} M and 10^{-9} M in water) in 90 x 15 mm Petri dishes. The solution should be prepared immediately before use.
2. Float leaf lamina segments on BL solution (see Recipes) (0 M, 10^{-6} M, 10^{-7} M, 10^{-8} M and 10^{-9} M) in an incubator at 29 °C in the dark for 2 days (Figure 3).

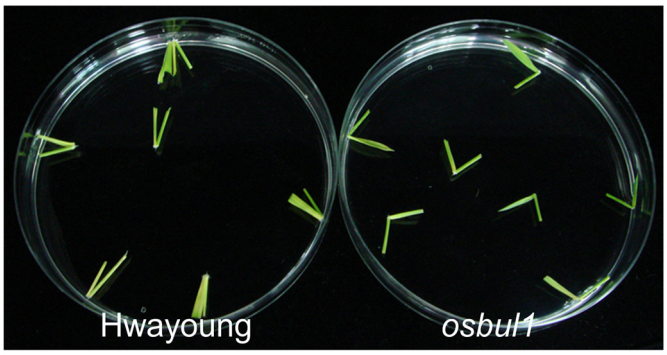

Figure 3. BR-induced lamina joint inclination in WT (Hwayoung cultivar) and *OsBUL1* knock-out mutant (*osbul1*) rice. The photo was taken after BL (10^{-6} M) treatment for 2 days.

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3. After 2 days, take sample photos with a digital camera, print them and measure the angle induced between the lamina and the sheath (degree of angle of leaf blade against the axis of leaf sheath) with a protractor (Figure 4).

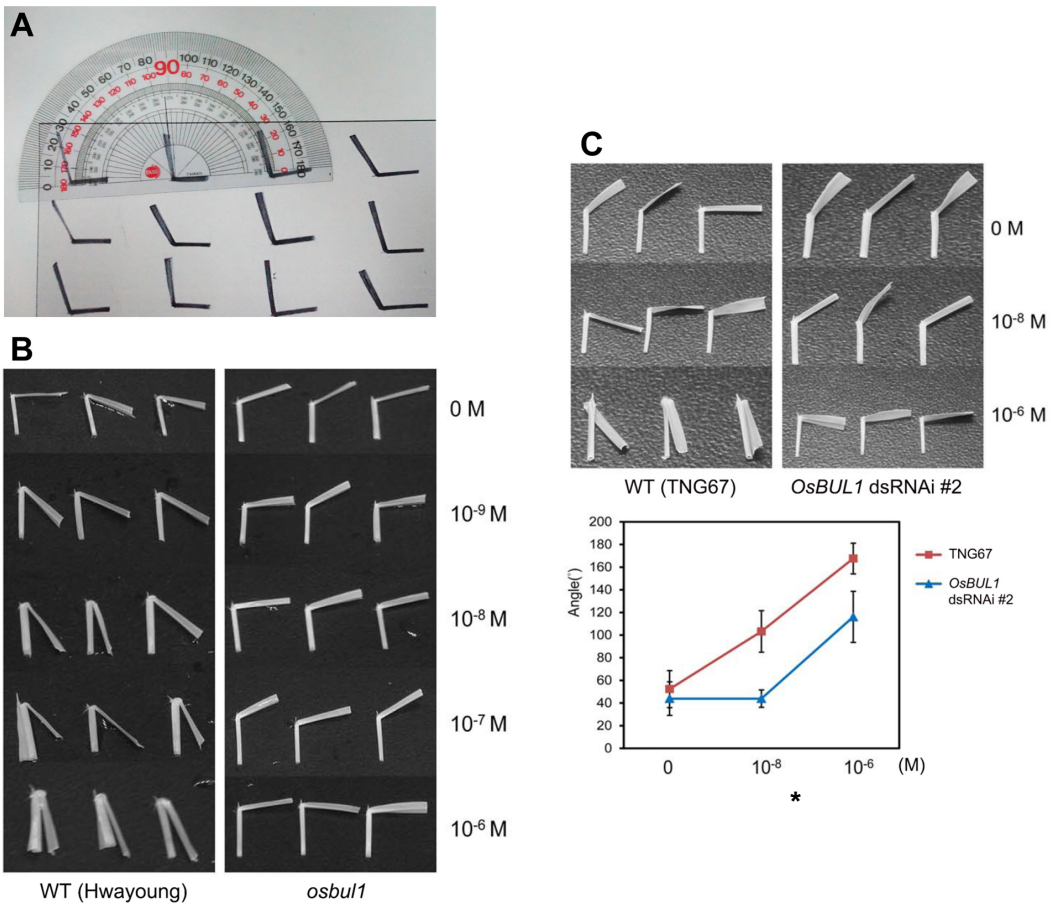

Figure 4. Measurement of lamina angles. A. Measuring the lamina angle with a protractor; B. Representative leaf segments containing lamina joint, blade and sheath from WT (Hwayoung cultivar) and *OsBUL1* KO (*osbul1*) plants after incubation for 48 h in the BL solution at different concentrations. C. Reduced leaf angles of *OsBUL1* dsRNAi line (#2) compared to WT (TNG67 cultivar) in 0 M, 10^{-8} M and 10^{-6} M BL solution (*$P < 0.001$).

Data analysis

The same number of rice seedlings was used for each BL concentration set (n = 6-12). When ImageJ software was used for lamina angle measurement, photo files containing rice lamina fragments were opened in the program. By using the angle tool in the software (https://imagej.nih.gov/ij/docs/tools.html), 3 points, one each for the lamina blade, joint and sheath in the lamina fragment were fixed and the angle made by the three points was measured (Figure 5). Measured angle values are presented as means with standard deviations using Microsoft Office

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2011 Excel (Figure 4C). The Student's *t*-test is used for statistical significance. Data from at least three independent repeats were obtained.

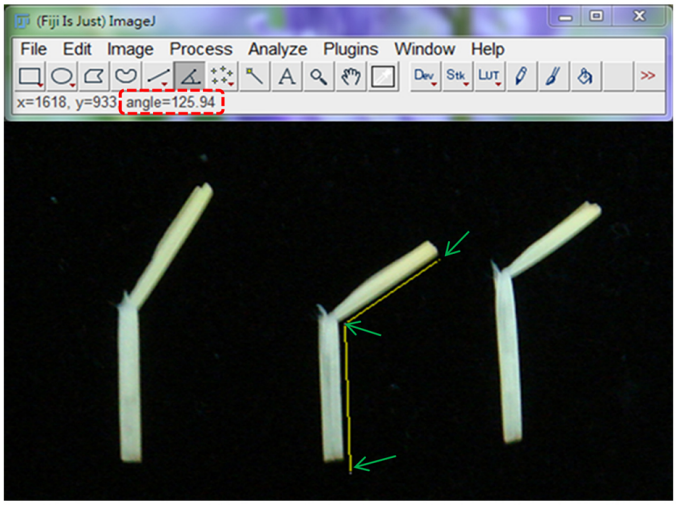

Figure 5. Measurement of lamina angles using the ImageJ program. The three points are marked by green arrows using the angle tool. The measured angle is marked by the box with a dashed red border.

Notes

BL solution should be freshly prepared. Since the sensitivity of this test varied within the rice cultivars employed, crossed data analyses with different rice cultivars should be avoided. For lamina angle measurement, image processing computer programs such as ImageJ can also be used.

Recipes

1. Sodium hypochlorite solution (with final available chlorine of 2%) for 30 ml
   Add 10 ml of commercial Bleach (CLOROX) into 20 ml of sterile water containing a few drops of Tween 20
2. 5 N potassium hydroxide (KOH) solution for 30 ml
   Add 8.417g KOH into 30 ml distilled water and filter the solution with a syringe filter
3. Murashige & Skoog (MS) media for 500 ml
   2.2 g Murashige & Skoog basal medium with Vitamins
   15 g sucrose
   Distilled water up to 500 ml

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Adjust pH to 5.7 with a few drops of 5 N KOH, and distribute 100 ml into a 600 ml beaker containing 0.3 g phytogel each, and then cover the beaker with a glass Petri dish. Seal the beaker with 3M micropore tape and autoclave

4. 1 mM Brassinolide (BL) stock solution
   a. Dissolve 2 mg of BL (molecular weight 480.68) in 1 ml of ethanol
   b. Add 3.16 ml of distilled water to get 1 mM final concentration
   c. Then split into aliquots in 1 ml tubes and store at -20 °C
   d. Serial dilution: *e.g.*, to make 10^{-4} M BL solution, 1 ml of BL (10^{-3} M) mixed with 9 ml of sterile water becomes 10 ml of 10^{-4} M BL solution

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