Chapter 5

Approaches to Study Light Effects on Brassinosteroid Sensitivity

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Abstract

Light perception and hormone signaling in plants are likely connected at multiple points. Light conditions, perceived by photoreceptors, control plant responses by altering hormone concentration, tissue sensitivity, or a combination of both. Whereas it is relatively straightforward to assess the light effects on hormone levels, hormone sensitivity is subjected to interpretation. In *Arabidopsis thaliana* seedlings, hypocotyl length is strongly affected by light conditions. As hypocotyl elongation also depends on brassinosteroids (BRs), assaying this response provides a valuable and easy way to measure the responsiveness of seedlings to BRs and the impact of light. We describe a simple protocol to evaluate the responsiveness of hypocotyls to commercial BRs and/or BR inhibitors under a range of light conditions. These assays can be used to establish whether light affects BR sensitivity or whether BRs affect light sensitivity. Overall, our protocol can be easily applied for deetiolation (under polychromatic or monochromatic light) and simulated shade treatments combined with BR treatments.

Key words Monochromatic light, Dark, Shade, Hypocotyl length, Deetiolation, Photoreceptors, Hormone sensitivity

1 Introduction

The development of young seedlings is determined by multiple environmental factors, of which light is a key signal. Light conditions are perceived by a battery of photoreceptors, such as the blue light-absorbing cryptochromes or the red/far-red light-absorbing phytochromes, that interpret these light signals and activate highly coordinated and regulated transcriptional networks. Young seedlings that germinate in the dark follow a developmental program known as skotomorphogenesis, characterized by elongated seedling stems (hypocotyls), unopened apical hooks, as well as closed cotyledons. Upon exposure to light, seedlings switch to a different developmental process, called photomorphogenesis, in which hypocotyl elongation is inhibited, the apical hook and the

Eugenia Russinova and Ana I. Caño-Delgado (eds.), *Brassinosteroids: Methods and Protocols*, Methods in Molecular Biology, vol. 1564, DOI 10.1007/978-1-4939-6813-8_5,

cotyledons open, and the seedlings become photosynthetically active. Exposure of emerging seedlings to light conditions that simulate vegetation proximity also promotes hypocotyl elongation and might inhibit cotyledon expansion [1, 2].

A central hub in the activation of the gene expression pattern required for the implementation of the corresponding developmental program is constituted by the PHYTOCHROME INTERACTING FACTORs (PIFs). These transcription factors are regulated by the circadian clock and temperature, and their stability and/or DNA-binding abilities are affected through interaction with phytochromes and cryptochromes [3, 4] that initiate and modulate a regulatory transcriptional network.

Brassinosteroids (BRs), like auxins and gibberellins, are a group of plant hormones that generally promote cell elongation and expansion. Overall, there are multiple contact points between light and hormone signaling that likely link light perception and the actual changes in plant growth and physiology. Specifically, BRs play an important role in photomorphogenesis and act as negative regulators of hypocotyl elongation and positive regulators of cotyledon expansion [5]. In *Arabidopsis thaliana*, the elongation of hypocotyls in the dark, under monochromatic light, or in response to plant proximity or shade requires BRs [6]. The light control of hormone-triggered responses might be exerted by concentration changes, alterations in tissue sensitivity, or a combination of both [7, 8]. Whereas measuring hormone levels is straightforward with the adequate analytical approaches, measuring hormone sensitivity is subjected to interpretation and is deduced from the responsiveness of specific tissues to exogenous hormones and/or plant growth regulators. It has been suggested that both hormone levels and sensitivity changes are required to promote hypocotyl elongation [7]. Furthermore, measuring the length of *Arabidopsis* hypocotyls is a valuable and simple way to measure the responsiveness of seedlings to light and BRs [5, 9], as well as to establish whether light affects BR sensitivity [8, 10, 11] or BRs affect light sensitivity [12].

This chapter provides a simple protocol for measuring the responsiveness of hypocotyls to different concentrations of commercial BRs and/or their inhibitors under various light conditions or regimes. We describe the use of different light conditions that can be easily tested to perform (1) deetiolation experiments using increasing amounts of polychromatic or monochromatic light, (2) simulated shade treatments, and (3) combinations of (1) and (2) with hormonal treatments.

2 Materials

2.1 Medium

1. Growth medium: half-strength Murashige and Skoog (½MS) mineral salts, 0.8 % (w/v) agar, and no sucrose (*see* **Note 1**). Mix 2.15 g MS salts and 0.8 g 2-(*N*-morpholino)ethanesulfonic acid (MES) and 900 mL water. Stir to dissolve. Adjust pH to 5.7 with KOH. Adjust volume with water to 1 L. Add 8 g Bacto agar. Sterilize by autoclaving.

2.2 Sterilization Solutions and Equipment

1. Imbibition solution: 0.1 % (v/v) polyoxyethylene sorbitan monolaureate (Tween 20).
2. Sterilization solution: 0.1 % (v/v) Tween 20, 10 % (v/v) sodium hypochlorite.
3. Sterile milliQ water.
4. Microcentrifuge tubes (1.5–2 mL).
5. Water bath.
6. Pasteur pipettes or standard blue pipette tips (200–1000 μL).
7. Sterile nylon mesh (100 μM, Nitex mesh).
8. Petri dishes (90 mm diameter and 16 mm height).
9. Porous surgical tape (e.g., tape 1.25 cm × 9.1 m).

2.3 Light Sources and Treatments

1. Aluminum foil for dark treatments (*see* **Note 2**).
2. Monochromatic blue (B), red (R) and far-red (FR) light provided by light-emitted diodes (LEDs) (for instance, GreenPower LED module HF blue, deep red, and far-red from Philips).
3. White light (W) provided by cool-white fluorescent bulbs (for instance, Philips TL-D 36w/840).
4. Filter paper (60 g/m^2) and grey filter to obtain different light intensities by placing several layers of neutral filters between the light sources and the seed-containing plates (*see* **Note 3**).
5. W supplemented with monochromatic FR LEDs to simulate shade treatments.
6. Spectroradiometer to measure fluence rates (such as a Spectrosense2 meter associated with a 4-channel sensor; Skye Instruments Ltd.) that quantifies photosynthetically active radiation (PAR) (400–700 nm) and 10 nm windows in the B (463–472 nm), R (664–673 nm), and FR (725–734 nm) regions.

2.4 Plant Growth Regulators

1. Prepare the biologically active BR, epibrassinolide (eBL), (CAS 78821-43-9) as a 5 mM stock solution in ethanol 50 % (v/v) and store it at −20 °C until use. It can be added to the MS

medium in a range of concentrations (from 0.01 to 10^{-6} M) and poured into plates.

2. Prepare the potent BR synthesis inhibitor brassinazole (BRZ) as a 10 mM stock solution in dimethoxysulfoxide (DMSO) and store it at −20 °C until use. It can be added to the MS medium in a range of concentrations (from 10 to 1000 nM) and poured into plates.
3. Prepare propiconazole (PCZ), the active ingredient of a broad-spectrum fungicide that acts as a BR synthesis inhibitor in both *Arabidopsis* and maize (*Zea mays*) seedlings [13] as a 5 mM stock solution in water and store it at −20 °C until use.

2.5 Software for Hypocotyl Measurements

1. ImageJ software (http://rsb.info.nih.gov/) to measure the hypocotyl lengths of the seedlings.

3 Methods

3.1 Seed Surface Sterilization

1. Aliquot the amount of seeds needed for the experiment in microcentrifuge tubes (1.5–2 mL) for sterilization. The weight of 100 *Arabidopsis* (accession Columbia-0) wild-type dry seeds is approximately 0.002 g (*see* **Notes 4–6**).
2. Add 1 mL of imbibition solution for at least 30 min.
3. After removal of the imbibition solution, add 1 mL of sterilization solution for 10 min (not more).
4. Remove the sterilization solution and wash the seeds with 1 mL of sterile water (5 times) before sowing. Different sowing methods will depend on the experimental treatment you plan to perform (*see* Subheading 3.3).

3.2 Preparation of Plates for Sowing Seeds

For long-term treatments and hypocotyl experiments, growth regulators are added to the 1/2MS medium after cooling it to 55 °C in a water bath (*see* **Notes 7–9**). *Arabidopsis* seedlings are grown in regular petri dishes containing approximately 25 mL of sterile growth medium (1/2MS).

3.3 Seed Sowing

1. For long-term treatments and hypocotyl experiments, sow the seeds directly on the solid medium one by one using a micropipette or a Pasteur pipette (*see* **Notes 10–14**).
2. For short hormone treatments and/or analysis of gene expression, sow the seeds on a sterile nylon mesh (*see* **Note 15**).
3. After sowing, seal the plates with a porous surgical tape (*see* **Note 16**).

3.4 Light Treatments of Seedlings for Hypocotyl Elongation Analysis

1. For stratification, keep the plates containing the sown seeds in the dark at 4 °C for 2–5 days. This stratification treatment breaks dormancy and synchronizes the germination of the seeds.
2. For the deetiolation experiments, induce the germination by a brief (0.5–3 h) W treatment. Then, wrap the plates with at least two layers of aluminum foil (*see* **Note 2**) and keep them in the dark at 22 °C for up to 24 h from the moment germination was induced (*see* **Note 17**). Thereafter, keep one plate in darkness at 22 °C for the whole treatment (this is the dark control treatment in which seedlings elongate to their maximum capacity) and transfer the remainder of the plates to the different polychromatic (W) or monochromatic (B, R, or FR) light conditions (Fig. 1). We measure the hypocotyl length on day 4 from germination.
3. For the simulated shade treatments, after stratification, put plates to germinate in continuous W at 22 °C. On day 2, maintain half the plates under W and transfer the other half to W+FR. We measure the hypocotyl length on day 7 from germination (after 5 days of differential light/shade treatment) (Fig. 2) (*see* **Note 17**).

3.5 Measurement of Hypocotyls

1. To measure hypocotyl length, lay seedlings flat on the agar plates.
2. Take digital pictures of the plates. Include a reference scale (*see* **Note 18**).
3. Measure hypocotyl length by analyzing the digital images of the seedlings with Image J or similar software (*see* Subheading 2.5).

4 Notes

1. Addition of sucrose to the medium interferes with the hypocotyl phenotypes in seedlings, therefore avoid it.
2. For the deetiolation experiments (Fig. 1), use at least a double layer of aluminum foil when keeping the plates in the dark (i.e., in the initial 24 h of darkness before exposing them to light or in the dark-grown control seedlings). This additional precaution will avoid even small amounts of light that might reach the seedlings through small pores of the aluminum foil, hence impairing the experiment.
3. We recommend preparing boxes with the filters (*see* Fig. 1d) to obtain more reproducible results instead of putting filters just on top of the plates.

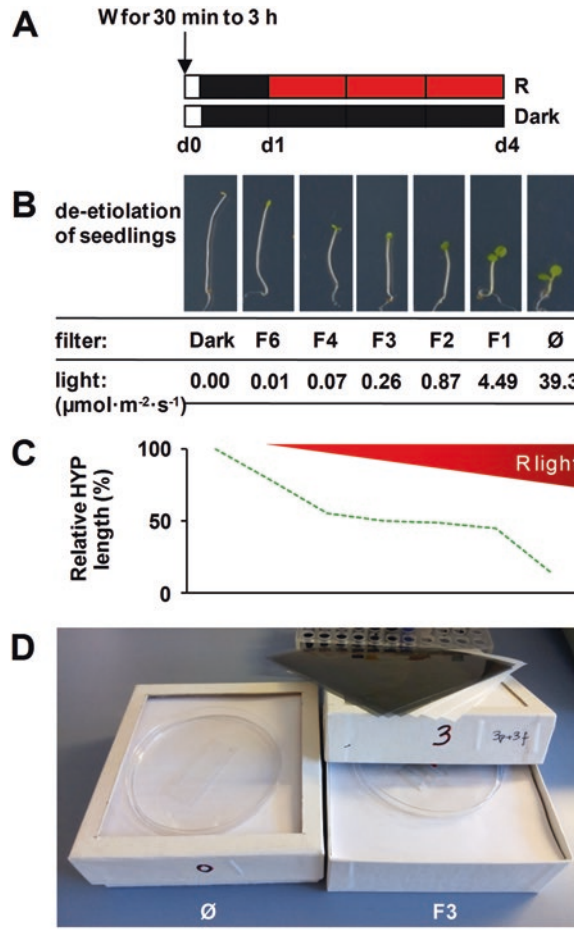

Fig. 1 Deetiolation of *Arabidopsis* seedlings under increasing amounts of light. (**a**) After induction of germination with a pulse of 2 h of white light (W), transfer of plates to the dark for 1 day followed by 3 days in monochromatic red light (R). As a control, a plate is kept in the dark. (**b**) Seedlings grown in the dark (*left*) or in increasing intensities of R (to the *right*). Dark-grown seedlings have long hypocotyls (HYP) and unopened apical hooks. R intensity is controlled by covering the plates with filters (F6-F1) that provide a range from 0.01 (F6) to 39.3 µmol·m^{-2}·s^{-1} without filter (Ø). (**c**) PhyB perception of R allowing transition from skotomorphogenesis to photomorphogenesis and decreasing hypocotyl lengths with increasing R intensity. (**d**) Example of boxes providing different light intensities. Box 3 (*right*) has three layers of grey filters and three layers of filter papers and corresponds to the F3 data (B). Box without filters (*left*) used for growing the seedlings in full R intensity, corresponds to Ø data (B)

4. When working with seedling phenotypes, always include the reference wild type together with the mutants of interest.
5. It is particularly important to compare seedlings from seeds originating from mother plants grown at the same time and under the same conditions, because any changes in seed age or

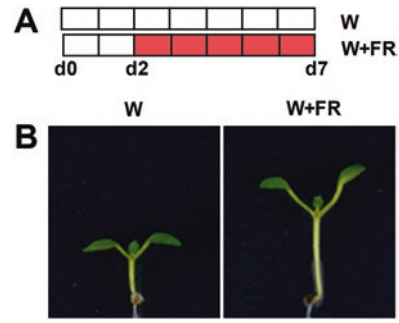

Fig. 2 Responses of *Arabidopsis* seedlings to simulated shade treatments. (**a**) *Arabidopsis* seedlings grown for 7 days (d) in continuous white light (W) or 2 days in W and 5 days in W supplemented with far-red light (W + FR). (**b**) Seedlings grown as indicated in a. Seedlings grown in W + FR show elongated hypocotyls, typical for shade avoidance

state could affect germination and, thus, influence the hypocotyl phenotypes.

6. For the hypocotyl measurements, use at least 20 seedlings for each data point and/or genotype. Repeat the experiments at least 3 times to help you carry out statistical analyses of the elongation data.
7. For experiments with hormones and inhibitors (Fig. 3), we recommend preparing serial dilutions of stocks for the different concentrations to test. The same stock can be used for different experiments as far as they are stored at −20 °C.
8. It is important to carefully homogenize the 1/2MS growth medium when hormones and/or inhibitors are added.
9. We normally use petri dishes of 90 mm diameter and 16 mm height (the same ones used to grow bacteria). Other sizes can also be employed, depending on the number of lines to compare.
10. After bleach sterilization and water washes, add 1 mL of sterile water. Seeds can be sown individually on top of the media with an automatic pipette and blue tips. Pull a small volume of water containing approximately 20 seeds. They will easily fall one by one just by touching the medium surface.
11. Some seed batches are too large to get inside the blue tips. In these cases, sterile Pasteur pipettes coupled with a rubber sucker can be used.
12. For light experiments, it is important to distribute seeds separately to avoid negative effects of elongation because of plant proximity or touching.
13. We normally sow about 100 seeds per petri dish, one by one. A plate can be divided in a maximum of four sections; a geno-

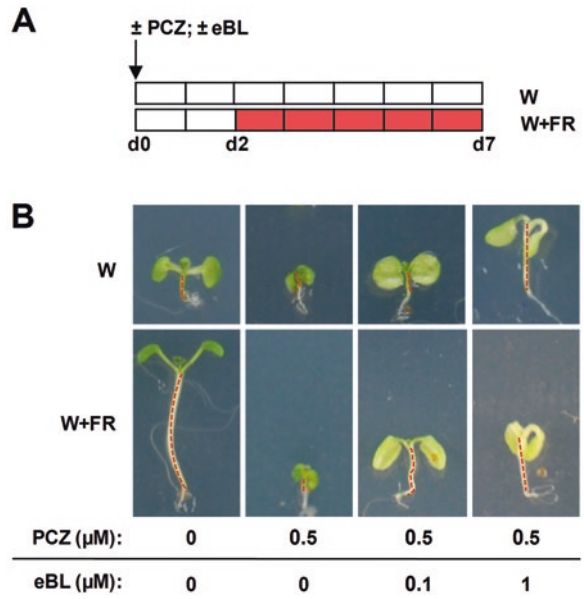

Fig. 3 Effect of simulated shade on the hypocotyl response to hormone application. (**a**) *Arabidopsis* Col-0 wild-type seedlings germinated and grown as indicated in Fig. 1a. Medium is supplemented from the day of sowing with propiconazole (PCZ) and with or without 24-epibrassinolide (eBL). (**b**) Seedlings grown as indicated (a) with the amounts of PCZ and eBL indicated below the images. *Red-dashed lines* mark hypocotyls of the representative seedlings grown as described

type is sown in each section (approximately 25 seeds per genotype).

14. Use a paper with a grid to sow regularly spaced seeds. Make sure any remaining water is completely evaporated before sealing the plates to start the stratification.
15. If you want to harvest material to analyze gene expression or protein extraction, sow the seeds on top of a nylon mesh (100 μM, Nitex Mesh) previously cut and sterilized. It will be easier and faster to harvest the plant material for further processing.
16. Usage of nonporous sealer (such as Parafilm or plastic wrap) is not recommended, because we have observed that hypocotyl elongation is clearly affected in such a closed atmosphere.
17. Be strict with the timing, especially for short light or hormone treatments. Perform the experiments always at the same time of the day, because circadian rhythms can influence the experiments. For instance, when plates are taken out from stratification to induce germination at 11 a.m., transfer half of the plates into W+FR at day 2 at 11 a.m. and end the experiment and take pictures on day 7 also at 11 a.m.

18. Remember to add a scale when taking the digital pictures of the hypocotyls, because it will be needed for measurement of the hypocotyl lengths.

Acknowledgments

SP received a predoctoral fellowship from the Catalan Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR, FI-DGR 2015). MJM-C received a predoctoral FPI fellowship from the Spanish Ministry of Economy and Competivity (MINECO). IR-V received a Beatriu de Pinós contract from AGAUR. Our research is supported by grants from the MINECO-FEDER (BIO2011-23489, BIO2014-59895-P) and AGAUR (2014-SGR447 and Xarba) to JFM-G.

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