Chapter 3

Physiological Analysis of Phototropic Responses in *Arabidopsis*

Mathias Zeidler

Abstract

Plants utilize light as sole energy source. To maximize light capture they are able to detect the light direction and orient themselves towards the light source. This phototropic response is mediated by the plant blue light photoreceptors phototropin1 and 2 (phot1 and phot2). Although fully differentiated plants also exhibit this response it can be best observed in etiolated seedlings. Differences in light between the illuminated and shaded site of a seedling stem lead to changes in the auxin-distribution, resulting in cell elongation on the shaded site. Since phototropism connects light perception, signaling, and auxin transport, it is of great interest to analyze this response with a fast and simple method.

Here we describe a method to analyze the phototropic response of *Arabidopsis* seedlings. With numerous mutants available, its fast germination and its small size *Arabidopsis* is well suited for this analysis. Different genotypes can be simultaneously probed in less than a week.

Key words Phototropism, *Arabidopsis*, Phototropin

1 Introduction

The first scientific observations of phototropic plant responses date back to the sixteenth century (reviewed in Ref. [1]). Darwin discovered that the perception of the stimulus and the site of action could be separated [2] which later led to the discovery of Auxin [3–5]. The responsible photoreceptors were identified with the help of molecular-genetics in the model plant *Arabidopsis thaliana* and later named after their major response: PHOTOTROPINS (phot) [6, 7]. *Arabidopsis* has two phots (phot1 and phot2) which are also involved in the regulation of chloroplast movement and stomatal opening. They are light-activated kinases. A sensory part with two LOV (light, oxygen, voltage) domains inactivates the kinase domain in the dark. After activation the sensory domain releases the kinase domain and phots can phosphorylate target proteins [8]. Phot1 regulates the phototropic response to a wide range

Paula Duque (ed.), *Environmental Responses in Plants: Methods and Protocols*, Methods in Molecular Biology, vol. 1398,
DOI 10.1007/978-1-4939-3356-3_3, © Springer Science+Business Media New York 2016

of blue light intensities while phot2 mainly mediates the response to high-intensity blue light [9].

Other photoreceptors modulate phot mediated phototropism, mainly the blue light sensing CRYPTOCHROMEs (cry1 and cry2; [10]) as well as the red light sensing phytochromes, especially PHYTOCHROME A (phyA) [11–14]. While crys and phyA appear to enhance the phototropic response to blue light, cryptochromes also seem to be responsible for residual phototropism in the *phot1phot2* double mutant [15]. Additionally phyA has been shown to mediate the enhancement of phototropism by a red light pretreatment [11, 16, 17]. Consequently a *cry1cry2phyA* triple mutant has a severely disturbed phototropic response [18]. Signaling factors for phototropism downstream of the phots have also been identified, among them NONPHOTOTROPIC HYPOCOTYL 3 (NPH3), ROOT PHOTOTROPISM 2 (RPT2) [19, 20], and the PHYTOCHROME KINASE SUBSTRATE (PKS) family with four members (PKS1–4). PKS1, PKS2, and PKS4 are associated with the plasma membrane and interact with phot1, phot2, and NPH3 [21–23]. PKS1, 2, and 4 as well as NPH3 have been shown to influence auxin distribution and auxin-dependentgene expression [24, 25]. Further downstream auxin transport facilitators are involved in phototropism. Among them are the PIN-FORMEDs (PINs), ATP-binding-cassette B-type transporter (ABCB), and the AUXIN RESISTANT (AUX) 1—LIKE-AUX (LAX) family proteins [26–31].

Many different techniques have been described to analyze phototropism in *Arabidopsis* seedlings from which the growth on vertical oriented agar plates is the most common and easiest, and therefore this technique is described here. Others, like continuous video documentation of the seedlings [32] or microbeam irradiation on single seedlings [33] have also been used successfully to measure hypocotyl bending in unilateral illumination conditions. They require a more sophisticated setup and data management but deliver time resolved information. Nevertheless, for the routine test of few lines the technique described here is easy and requires no specific instruments or software. Results can be obtained in less than a week.

2 Materials

1. 20–40 *Arabidopsis* seeds per line you want to investigate.
2. ½ MS solid medium: 2.15 g/l MS (Murashige–Skoog salts, without vitamins; *see* **Note 1**) in H_2O (Milli-Q). Adjust pH to 5.7 with KOH, add 0.7 % agar, and autoclave. Pour in sterile square petri dishes (12.5 × 12.5 × 1.5 cm).

3. Sterilization solution: 2.5 % (v/v) sodium hypochlorite in H_2O (Milli-Q) with 0.1 % (v/v) Triton X-100. Prepare fresh and be careful because the solution is quite corrosive.
4. Plating Solution: 0.1 % agarose (*see* **Note 2**) in H_2O (Milli-Q), autoclaved.
5. A narrow band width light source (full width at half height; FWHH < 30 nm) with the option to adjust different fluence rates is necessary. Usually a slide projector with an interference filter can be used. Much better are high power LEDs that are easily available. For unilateral blue light we use 450 nm Luxeon high power LEDs (Roithner Lasertechnik, Austria), which are mounted on a 2-mm thick aluminum plate for cooling and connected to a power supply. Fluence rates between 1 and 100 μmol m^{-2} s^{-1} can be easily obtained by changing the current, and for lower fluence rates semitransparent paper is brought into the light path.
6. Black paper.
7. Book stands or black wooden boxes in which up to 6 square petri dishes will fit (inner dimensions $10 \times 13 \times 13$ cm one side open).
8. Computer with the ImageJ software (National Institutes of Health) installed.

3 Methods

3.1 Seed Preparation

1. You will need 1–2 petri dishes per genetic line to investigate. Take the plates with the solidified media, turn them bottom-up and draw a line on the bottom of the petri dishes. The start point should be 5 mm before the top-midpoint of the plate and the end point 5 mm after the bottom midpoint of the plate if it lies on a table in front of you (Fig. 1a; *see* **Note 3**). Turn the plates back to the normal position (bottom-down).
2. Sterilize the seeds by mixing them with 500 μl of sterilization solution in 1.5-ml reaction tubes and let the suspension incubate for 10 min at room temperature (*see* **Note 4**). Centrifuge briefly. From this point onwards work on a sterile bench. Discard the sterilization solution quickly and wash the seeds three times with 1 ml of sterile water. After the last wash, discard the water, add 100 μl of 0.1 % agarose, and mix.
3. Pipette some of the seeds in 0.1 % agarose on the drawn line on the MS medium (Fig. 1a). Move the single seeds with a sterile inoculation loop. Make sure that the distance between each seed is approximately 3–5 mm (*see* **Note 5**). Let the surface of the medium dry briefly on the clean bench so that the seeds do not shift when the plates are placed in the vertical

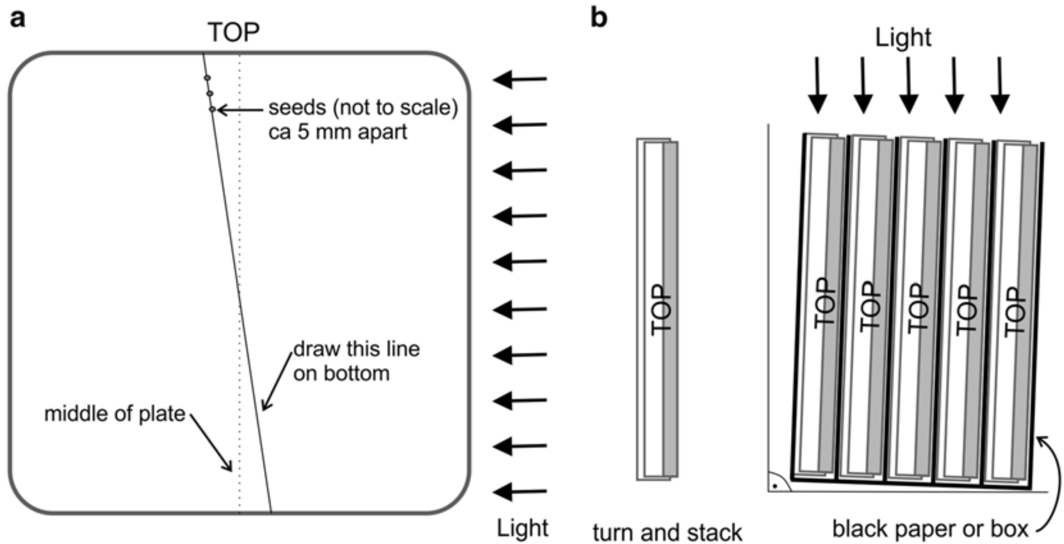

Fig. 1 Schematic drawings of plate layout and light path to the plate stack. (**a**) View of open plate with the line on the bottom slightly rotated in relation to the center of the plate. Seeds are separately arranged on this line with a distance of 3–5 mm each. (**b**) Plate is closed and placed vertically so that the TOP (see also **a**) is on the upper side. Plates are stacked with black paper in between them as well as on the outside and on the back of the stack. The stack is slightly turned so that the light falls with a 0.5–1° angle on the agar surface

position. Close the petri dishes and label them on the bottom with the genotype of the seeds they contain.

4. Stratify your seeds for 3–4 days wrapped in aluminum foil at 4 °C, already in the vertical position. Mark the top side of the plates.

3.2 Germination and Illumination

1. Unwrap the plates and expose 2 h to white light to synchronize germination.
2. Place the plates back in standing position and insert a black paper between each separate plate to minimize reflection. Also put a black paper at the back of the plate stack.
3. The plates should now be in a stack in which all the plates are standing, with black paper on both sides of the plates and on the back of the cube-like stack (Fig. 1b; *see* **Note 6**).
4. Let seeds germinate and the emerging seedlings grow negatively gravitropically for 2–3 days in darkness at 20–25 °C. Ideally, the plates should already be in front of the irradiation setup. The irradiation setup and position of the plate stack should be prepared in a dark room or in a completely dark cabinet.
5. After the germination and negative gravitropic growth, switch the light on to irradiate the seedlings from one side with blue light of the desired fluence rate for 12 h (*see* **Note 7**). The plates should be slightly moved out of the direct straight line

of the light path so that the light falls obliquely onto the agar surface (Fig. 1b). Depending on the light source, different fluence rates can be adjusted in the light field by shading the front side of the petri dish cubes with semitransparent paper.

6. Document the phototropic bending angle by digital photography. Place a piece of millimeter paper next to the seedlings to obtain a scale.

3.3 Measuring the Bending Angle with ImageJ

1. Install ImageJ from http://rsb.info.nih.gov/ij/ and start the program.
2. Open your image: File>Open.
3. Make sure the plate is aligned with the border of the window: Image>Transform>Rotate.
4. Change the image to 8-bit greyscale: Image>Type> 8-bit.
5. Click on the angle-measuring button in the tools menu.
6. Click once on the base of the hypocotyl, once on the midpoint of the bend and last on the upper part of the hypocotyl.
7. Press M on the keyboard to save the measurement in the results windows.
8. Go back to **step 6**.
9. When finished with one biological sample, copy the data to Excel or a similar program to plot the angles (*see* **Note 8**).

4 Notes

1. Adjust the amount of MS salts according to the recommendations of the supplier. The number here refers to MS-salts from Genaxxon (Germany).
2. Agarose is used here and not agar. The low concentration agarose has a similar density as the seeds and allows them to float. This makes pipetting and manipulating the seeds much easier.
3. A slightly slanted line is intended so that the seedlings do not disturb each other when growing negatively gravitropically.
4. It is important not to exceed these 10 min because then the seeds suffer damage and germination rates drop. On the other hand, a much shorter time does not sterilize the surface sufficiently. Therefore 10 min should be observed closely (+/– 1 min).
5. This may be difficult at the beginning because the seeds tend to stick to each other in the drops of liquid. Therefore, less liquid is better. Try first to position a few seeds and when you are done, take more out of the tube onto the agar surface.
6. Care should be taken that the plates are exactly upright. This can be achieved by constructing wooden boxes or by using bookstands on both sides of the upright stack.

7. For standard tests a fluence rate of 1 μmol m^{-2} s^{-1} should result in a good response of the wild type. Fluence rates should be determined with a PAR meter or similar device at the point where the seedlings will be positioned.
8. Usually it is not conclusive to take a mean value of the angles, especially if plants are not behaving normally (for instance showing a positive or negative reaction towards the light source). It therefore is better to take classes of angles (0–10°, 11–20° etc.) and sort all the measured values into these classes, so that a table is generated which comprises numbers of seedlings per class. This table needs to be normalized to the number of seedlings by translating the numbers per class to percentage of all seedlings per class. This dataset contains more information than the mean (Fig. 2).

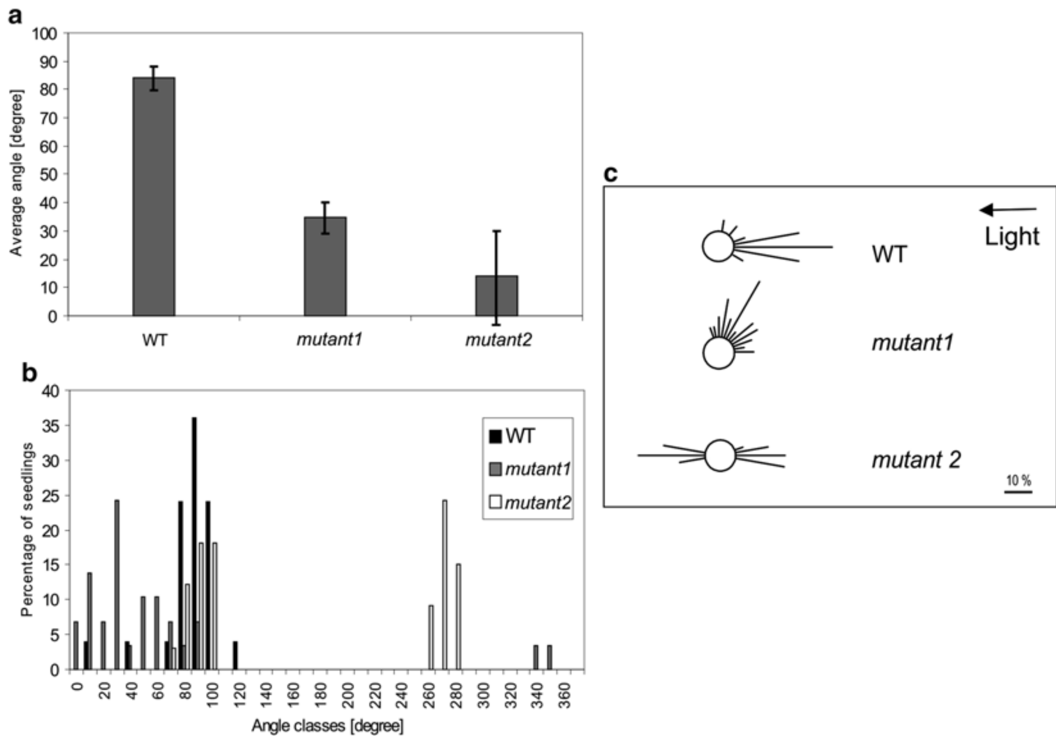

Fig. 2 Different presentations of the same bending angle data. Three artificial data sets, representing a normal distribution, a wide distribution, and an avoidance component in the bending response were plotted with three different methods. (**a**) An average does not differentiate between *mutant1* and *mutant2* (positive and negative phototropism) nor does it reflect the avoidance reaction. *Error bars* represent standard errors of the mean. (**b**) All seedlings were sorted into classes and normalized to the total amount of seedlings from each genotype. This is a much better representation of the response and the differences between the different genotypes. In (**c**) the same data were converted into circular histograms (*wuschel*-diagrams). Here the length of a line represents the percentage of seedlings in this specific angle class. This diagram, also based on the same data set as in **b**, is more informative. The light was coming from the right hand side as indicated by the *arrow* and the scale bar is 10 % of the seedlings represented

Acknowledgements

This work was supported by DFG grant ZE485/2-2 to MZ. I thank Anna Lena Lichtenthäler and Henrik Johansson for critical reading of the manuscript.

References

1. Whippo CW, Hangarter RP (2006) Phototropism: bending towards enlightenment. Plant Cell 18(5):1110–1119. doi:10.1105/tpc.105.039669
2. Darwin C (1880) The power of movements in plants. John Murray, London
3. Went FW (1926) On growth accelerating substances in the coleoptile of Avena sativa. Proc K Akad Wet 30:10–19
4. Cholodny N (1927) Wuchshormone und Tropismen bei den Pflanzen. Biol Zentralbl 47:604–626
5. Kögel F, Haagen-Smit AJ (1931) Mitteilung über pflanzliche Wachstumsstoffe. Über die Chemie des Wuchsstoffes. Proc K Ned Akad Wet 34:1411–1416
6. Huala E, Oeller PW, Liscum E, Han IS, Larsen E, Briggs WR (1997) *Arabidopsis* NPH1: a protein kinase with a putative redox-sensing domain. Science 278(5346):2120–2123
7. Sakai T, Kagawa T, Kasahara M, Swartz TE, Christie JM, Briggs WR, Wada M, Okada K (2001) *Arabidopsis* nph1 and npl1: blue light receptors that mediate both phototropism and chloroplast relocation. Proc Natl Acad Sci U S A 98(12):6969–6974
8. Okajima K, Matsuoka D, Tokutomi S (2011) LOV2-linker-kinase phosphorylates LOV1-containing N-terminal polypeptide substrate via photoreaction of LOV2 in *Arabidopsis* phototropin1. FEBS Lett 585(21):3391–3395
9. Kagawa T, Sakai T, Suetsugu N, Oikawa K, Ishiguro S, Kato T, Tabata S, Okada K, Wada M (2001) *Arabidopsis* NPL1: a phototropin homolog controlling the chloroplast high-light avoidance response. Science 291(5511):2138–2141
10. Ahmad M, Jarillo JA, Smirnova O, Cashmore AR (1998) The CRY1 blue light photoreceptor of *Arabidopsis* interacts with phytochrome A in vitro. Mol Cell 1(7):939–948
11. Parks BM, Quail PH, Hangarter RP (1996) Phytochrome A regulates red-light induction of phototropic enhancement in *Arabidopsis*. Plant Physiol 110(1):155–162
12. Lariguet P, Fankhauser C (2004) Hypocotyl growth orientation in blue light is determined by phytochrome A inhibition of gravitropism and phototropin promotion of phototropism. Plant J 40(5):826–834
13. Rösler J, Klein I, Zeidler M (2007) *Arabidopsis fhl/fhy1* double mutant reveals a distinct cytoplasmic action of phytochrome A. Proc Natl Acad Sci U S A 104(25):10737–10742
14. Rösler J, Jaedicke K, Zeidler M (2010) Cytoplasmic phytochrome action. Plant Cell Physiol 51(8):1248–1254
15. Ohgishi M, Saji K, Okada K, Sakai T (2004) Functional analysis of each blue light receptor, cry1, cry2, phot1, and phot2, by using combinatorial multiple mutants in *Arabidopsis*. Proc Natl Acad Sci U S A 101(8):2223–2228
16. Hangarter RP (1997) Gravity, light and plant form. Plant Cell Environ 20(6):796–800
17. Janoudi AK, Gordon WR, Wagner D, Quail P, Poff KL (1997) Multiple phytochromes are involved in red-light-induced enhancement of first-positive phototropism in *Arabidopsis thaliana*. Plant Physiol 113(3):975–979
18. Tsuchida-Mayama T, Sakai T, Hanada A, Uehara Y, Asami T, Yamaguchi S (2010) Role of the phytochrome and cryptochrome signaling pathways in hypocotyl phototropism. Plant J 62(4):653–662
19. Motchoulski A, Liscum E (1999) *Arabidopsis* NPH3: a NPH1 photoreceptor-interacting protein essential for phototropism. Science 286(5441):961–964
20. Sakai T, Wada T, Ishiguro S, Okada K (2000) RPT2. A signal transducer of the phototropic response in *Arabidopsis*. Plant Cell 12(2):225–236
21. Lariguet P, Schepens I, Hodgson D, Pedmale UV, Trevisan M, Kami C, de Carbonnel M, Alonso JM, Ecker JR, Liscum E, Fankhauser C (2006) PHYTOCHROME KINASE SUBSTRATE 1 is a phototropin 1 binding protein required for phototropism. Proc Natl Acad Sci 103(26):10134–10139
22. de Carbonnel M, Davis P, Roelfsema MR, Inoue S, Schepens I, Lariguet P, Geisler M,

Shimazaki K, Hangarter R, Fankhauser C (2010) The *Arabidopsis* PHYTOCHROME KINASE SUBSTRATE2 protein is a phototropin signaling element that regulates leaf flattening and leaf positioning. Plant Physiol 152(3):1391–1405

23. Demarsy E, Schepens I, Okajima K, Hersch M, Bergmann S, Christie J, Shimazaki K, Tokutomi S, Fankhauser C (2012) Phytochrome kinase substrate 4 is phosphorylated by the phototropin 1 photoreceptor. EMBO J 31(16):3457–3467
24. Haga K, Takano M, Neumann R, Iino M (2005) The Rice COLEOPTILE PHOTOTROPISM1 gene encoding an ortholog of *Arabidopsis* NPH3 is required for phototropism of coleoptiles and lateral translocation of auxin. Plant Cell 17(1):103–115
25. Kami C, Allenbach L, Zourelidou M, Ljung K, Schutz F, Isono E, Watahiki MK, Yamamoto KT, Schwechheimer C, Fankhauser C (2014) Reduced phototropism in pks mutants may be due to altered auxin-regulated gene expression or reduced lateral auxin transport. Plant J 77(3):393–403. doi:10.1111/tpj.12395
26. Friml J, Wisniewska J, Benkova E, Mendgen K, Palme K (2002) Lateral relocation of auxin efflux regulator PIN3 mediates tropism in *Arabidopsis*. Nature 415(6873):806–809. doi:10.1038/415806a
27. Ding Z, Galvan-Ampudia CS, Demarsy E, Langowski L, Kleine-Vehn J, Fan Y, Morita MT, Tasaka M, Fankhauser C, Offringa R, Friml J (2011) Light-mediated polarization of the PIN3 auxin transporter for the phototropic response in *Arabidopsis*. Nat Cell Biol 13(4):447–452. doi:10.1038/ncb2208
28. Noh B, Murphy AS, Spalding EP (2001) Multidrug resistance-like genes of *Arabidopsis* required for auxin transport and auxin-mediated development. Plant Cell 13(11): 2441–2454
29. Nagashima A, Uehara Y, Sakai T (2008) The ABC subfamily B auxin transporter AtABCB19 is involved in the inhibitory effects of N-1-naphthyphthalamic acid on the phototropic and gravitropic responses of *Arabidopsis* hypocotyls. Plant Cell Physiol 49(8):1250–1255. doi:10.1093/pcp/pcn092
30. Christie JM, Yang H, Richter GL, Sullivan S, Thomson CE, Lin J, Titapiwatanakun B, Ennis M, Kaiserli E, Lee OR, Adamec J, Peer WA, Murphy AS (2011) phot1 inhibition of ABCB19 primes lateral auxin fluxes in the shoot apex required for phototropism. PLoS Biol 9(6):e1001076. doi:10.1371/journal.pbio.1001076
31. Stone BB, Stowe-Evans EL, Harper RM, Celaya RB, Ljung K, Sandberg G, Liscum E (2008) Disruptions in AUX1-dependent auxin influx alter hypocotyl phototropism in *Arabidopsis*. Mol Plant 1(1):129–144. doi:10.1093/mp/ssm013
32. Whippo CW, Hangarter RP (2003) Second positive phototropism results from coordinated Co-action of the phototropins and cryptochromes. Plant Physiol 132(3):1499
33. Yamamoto K, Suzuki T, Aihara Y, Haga K, Sakai T, Nagatani A (2014) The phototropic response is locally regulated within the topmost light-responsive region of the *Arabidopsis thaliana* seedling. Plant Cell Physiol 55(3):497–506. doi:10.1093/pcp/pct184