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Highlights

- The upper hypocotyl perceives the phototropic stimulus in *Arabidopsis* seedlings

- Cotyledons and the hypocotyl apex are not necessary for phototropism in *Arabidopsis*

- Local activation of phototropin 1 leads to a rapid global response

- Spatial aspects of signaling during phototropism differ between grasses and dicots

Title page

Defining the site of light perception and initiation of phototropism in *Arabidopsis*.

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Running title: Site of light perception for phototropism.

Keywords: phototropism, phototropin 1, blue light perception, *Arabidopsis thaliana*.

Summary

Phototropism is an adaptive response allowing plants to optimize photosynthetic light capture [1–7]. This is achieved by asymmetric growth between the shaded and lit sides of the stimulated organ [8, 9]. In grass seedlings, the site of phototropin-mediated light perception is distinct from the site of bending [10–12]; however, in dicotyledonous plants (e.g. *Arabidopsis*), spatial aspects of perception remain debatable. We use morphological studies and genetics to show that phototropism can occur in the absence of the root, lower hypocotyl, hypocotyl apex and cotyledons. Tissue-specific expression of the phototropin1 (phot1) photoreceptor [13] demonstrates that light sensing occurs in the upper hypocotyl and that expression of phot1 in the hypocotyl elongation zone is sufficient to enable a normal phototropic response. Moreover we show that efficient phototropism occurs when phot1 is expressed either from endodermal, cortical or epidermal cells and that its local activation rapidly leads to a global response throughout the seedling. We propose that spatial aspects in the steps leading from light perception to growth re-orientation during phototropism differ between grasses and dicots. These results are important to properly interpret genetic experiments and establish a model connecting light perception to the growth response, including cellular and morphological aspects.

Results and Discussion

Perception of a phototropic light stimulus and phototropic bending do not require cotyledons and the hypocotyl apex

Plants are photoautotrophic and therefore depend on the energy of sunlight to sustain growth and reproduction. Phototropic reorientation contributes to the optimization of photosynthesis particularly in low light environments [1–7]. In order to understand the process of phototropism, spatial considerations are essential and have been investigated since the pioneering experiments of Charles Darwin (reviewed in [9, 14]). However, these and later studies were predominantly conducted on monocotyledonous (monocot) grasses. Since the establishment of *Arabidopsis* as a model species, a large body of molecular information has been gathered in this dicotyledonous (dicot) plant. The divergence of monocots and dicots occurred ~140 to 150 million years ago [15, 16] and the morphology of dicot seedlings is fundamentally different from that of grass seedlings. While the pin-like structure of grass seedlings, known as coleoptile, consists of a rigid sheath surrounding and protecting the first leaf, dicot seedlings possess a hypocotyl (the embryonic stem) sustaining the cotyledons.

For grass coleoptiles light perception in the most apical tip triggers the formation of an auxin gradient leading to asymmetric growth further down, i.e. the sites of signal perception and phototropic bending are distinct [10–12]. Similarly, the light signal for negative phototropism in maize roots is perceived in the root cap, while curvature takes place in the central elongation zone [17]. In contrast, spatial aspects of the steps leading to phototropism in hypocotyls of dicots are considerably less well understood and over the years signal

perception has been assigned to either leaves or cotyledons [18, 19], or the responding organ itself [20–23].

Hypocotyl phototropism is typically studied in etiolated seedlings (both in grasses and dicots ; see [12]) as upon emergence from the soil the seedling may have to grow towards a favorable light source. However de-etiolated seedlings also encounter situations triggering phototropism. A recent study on de-etiolated *Arabidopsis* seedlings reports evidence for a conserved topology between the sites of light perception and phototropic bending in angiosperms with lateral auxin fluxes being initiated at and above the hypocotyl apex [24]. The authors furthermore suggest that the cotyledons do not play a major role in the phototropic response, which is in accordance with earlier reports [12, 20, 21, 23, 25] and is also clearly supported by our present study using decapitated wild-type seedlings and mutants lacking cotyledons (Fig. 1). Furthermore, our experiments show that in etiolated *Arabidopsis* seedlings it is not the hypocotyl apex but the region in and above the elongation zone that is necessary for phototropism. When we removed cotyledons and the apex (position 1, Fig. 1A), seedlings showed phototropic bending comparable to untreated seedlings, whereas decapitation within or below the elongation zone (positions 2 and 3, respectively) prevented any response to the light stimulus (Fig. 1, Fig. S1). Interestingly, amputated upper parts of seedlings that contained cotyledons, intact apical hooks, and elongation zones responded to the light stimulus (Fig. 1A, Fig. S1D). We thus conclude that in etiolated seedlings the upper hypocotyl (containing the elongation zone) is sufficient to induce phototropism. In order to evaluate the role of the hypocotyl apex in light-grown seedlings we analyzed phototropism in de-etiolated seedlings lacking cotyledons and the hypocotyl

apex (Fig. S1G). These seedlings still grew towards the light; however, the response was not as robust as in etiolated seedlings (Fig. S1), suggesting a greater role of the cotyledonary node and apical meristem in de-etiolated than etiolated seedlings [24].

As decapitation experiments induce a wounding response that may influence phototropism, we next analyzed *Arabidopsis* seedlings carrying the *pidwag1wag2* triple mutation [26]. These seedlings do not develop cotyledons, [26] thereby providing a system to study the importance of cotyledons during phototropism without mechanically manipulating the plant. Homozygous *pidwag1wag2* triple mutants showed pronounced phototropic bending (Fig. S1E), although bending rates were slightly slower compared to siblings with normal cotyledons (Fig. 1C). Together, our results show that cotyledons, the hypocotyl apex, and all tissue below the elongation zone are not required for perception of a phototropic light stimulus and establishment of phototropic curvature in etiolated *Arabidopsis* seedlings.

Phototropism requires cell elongation, thus an altered phototropic response might result from a general growth defect. We found that seedlings cut at position 2 showed almost no growth during six hours of illumination while seedlings cut at position 1 displayed robust growth, although significantly less than untreated seedlings under these conditions (Fig 1B). The *pidwag1wag2* triple mutant also showed reduced elongation growth (Fig. 1D). Sustained growth of seedlings cut at position 1 is noteworthy, as phototropism depends on the asymmetric distribution of auxin (reviewed in [8, 9, 27]), which is primarily synthesized in cotyledons and the apical meristem [28]. It has previously been demonstrated that removal of these parts leads to strongly reduced hypocotyl

growth [29, 30]. We therefore analyzed seedlings decapitated in the apical hook (position 1) 90 minutes prior to blue light irradiation. Such seedlings still displayed a robust phototropic response, but reduced compared to untreated samples (Fig. S1A). Interestingly, the rates of bending and growth were similar in untreated and decapitated seedlings early during phototropic reorientation, while both rates later declined drastically in decapitated seedlings (Fig. S1A-C).

The more pronounced impairment of growth rate and phototropism of decapitated seedlings compared to *pidwag1wag2* (compare Fig. 1 and S1) may be due to the removal of more auxin-producing tissue by decapitation. An alternative hypothesis for reduced phototropism in seedlings lacking cotyledons might be a reduced photoperceptive area (absence of cotyledons that express phot1). However, selective expression of phot1-mCitrine in the hypocotyl elongation zone perfectly restores phototropism in *phot1phot2* (Fig. 2), suggesting that the reduced phototropic response in decapitated seedlings is not due to a reduction in the photoperceptive area. Taken together, our results suggest that phototropism in etiolated *Arabidopsis* hypocotyls does not require auxin coming from cotyledons, the hypocotyl apex or lower parts. We thus propose that local redistribution of auxin within the upper hypocotyl, below the cotyledonary node, is sufficient to promote phototropism.

The modest phototropic phenotype of *pidwag1wag2* mutants is somewhat surprising given that these protein kinases regulate the polarization of PIN-FORMED (PIN) proteins and thus polar auxin transport [25, 31, 32]. The phenotype was previously interpreted as resulting from reduced lateral polarization of PIN3 in the hypocotyl elongation zone [25]. An alternative hypothesis is that the slow phototropic response in *pidwag1wag2* results either

from a reduced supply of auxin in the hypocotyl elongation zone (Fig. 1) or from a combination of both effects.

Local activation of phot1 in any cell layer within the upper hypocotyl leads to a rapidly spreading signal to promote phototropic bending

While in coleoptiles phot1 is mainly expressed in the tip, where light perception and initiation of the phototropic response take place [11], its expression is much broader in etiolated *Arabidopsis* seedlings [33, 34]. To narrow down the region essential for perception of the phototropic stimulus, we generated transgenic *Arabidopsis* lines expressing fluorescently-tagged phot1 under the control of different promoters in a *phot1phot2* background. We selected experimental conditions (etiolated seedlings irradiated with low fluence-rate blue light) under which phot1 alone controls phototropism. Thus we did not analyze the role of phot2 in this work [2, 34, 35] (Fig. S2).

Expressed under its endogenous promoter (*pPHOT1*) [33], phot1-mCitrine was able to restore phototropism in a *phot1phot2* double mutant background (Fig. 2B), thus showing that the fusion-protein is functional. In accordance with an earlier publication [33], we found that phototropic bending in the transformants was slightly but significantly slower compared to the wild-type (Fig. 2B). Using multiple independent lines with different expression levels of phot1 we were able to show that limited amounts of phot1 are sufficient to trigger the full phototropic response without any obvious dose effect (Fig. S2B).

Selective expression of phot1-mCitrine allowed us to show that local activation of the photoreceptor in the upper hypocotyl is necessary to promote phototropism (Fig. 2). Seedlings expressing phot1-mCitrine only in the root and

lower third of the hypocotyl (*CASPARIAN STRIP MEMBRANE DOMAIN PROTEIN 1* promoter [36], *pCASP1*; Fig. S3) remained aphototropic, while focused expression of the photoreceptor in the elongation zone, but not in more apical parts (*PHYTOCHROME KINASE SUBSTRATE 4* promoter [37], *pPKS4*; Fig. S3) fully restored phototropic bending (Fig. 2B). Furthermore, phototropism could be restored when phot1-mCitrine was exclusively expressed in cotyledons and the apical hook (*CHLOROPHYLL A/B BINDING PROTEIN 3* promoter [38], *pCAB3*; Fig. 2B, Fig. S3C).

As discussed above, cotyledons and the apical meristem are dispensable for phototropism (Fig. 1). We thus propose either that perception in the rather small overlap of *pCAB3* and *pPKS4* expression domains (only a few cells in the lower apical hook region) is sufficient to trigger the full phototropic response or that perception can occur over a quite broad area, including the hypocotyl elongation zone, apical hook, hypocotyl apex, and cotyledons. We note that perception of a light gradient in the cotyledons and the hypocotyl apex would be complex in etiolated dicot seedlings emerging into the light. Indeed, light also triggers opening of the apical hook and unfolding of the cotyledons, thereby altering the position of those organs relative to the hypocotyl that undergoes phototropic reorientation. The anatomical differences between grasses and dicots may therefore underlie the mechanistic differences in phototropin signaling uncovered in this study.

It has previously been shown that phot1 is expressed in all cell layers of *Arabidopsis* hypocotyls [33, 34]. This raised the question as to where exactly phot1 is activated in response to a phototropic stimulus. For this purpose we introduced phot1-mCitrine translational fusion constructs driven by promoters

of *MERISTEM LAYER 1* (*ATML1*) for epidermal [39], *AT1G09750* (*C1*) for cortical [40], and *SCARECROW* (*SCR*) for endodermal expression [41] (Fig. 3A) into the *phot1phot2* background. The three tissue-specific promoters led to expression of phot1-mCitrine in the upper hypocotyl, including the elongation zone and apical hook and showed the expected cell-type specificity (Fig. S3).

Interestingly, the expression of phot1-mCitrine in any single tissue within the upper hypocotyl region of etiolated seedlings is able to fully complement the *phot1phot2* mutant (Fig. 3B). Phototropism was also restored when de-etiolated seedlings were used for the experiments even if rates of phototropic bending were generally reduced in de-etiolated compared to etiolated seedlings, especially in the wild-type (Fig. S3D). This is somewhat surprising, as the establishment of an auxin gradient and subsequent asymmetric growth supposedly involve responses in different cell layers (e.g. relocalization of PIN3 in the endodermis [42], cell growth especially in the epidermis as growth promoting and restricting tissue [39, 43], basipetal auxin transport in the vasculature and the epidermis [24]). A possible explanation is that phot1 activation triggers a rapid signaling cascade that spreads over the different cell layers within the hypocotyl. To test this hypothesis we examined the modification of NON-PHOTOTROPIC HYPOCOTYL 3 (NPH3), a protein that is crucial for phototropism [44–46] and has been proposed to act as a signal transducer between light perception and the establishment of an auxin gradient [11, 47]. Upon blue-light irradiation NPH3 is quickly dephosphorylated in a phot1-dependent manner [48]. However, the importance of this dephosphorylation for phototropism is still unclear [48, 49]. As NPH3 is broadly expressed throughout the hypocotyl [7] (Fig. S4), we tested whether local

activation of phot1 leads to global changes in the NPH3 phosphorylation state. We found that the rates of NPH3 dephosphorylation are very similar for seedlings expressing phot1 throughout the hypocotyl and seedlings exclusively expressing the photoreceptor in a single cell type (Fig. 4, Fig. S4). We thus conclude that local activation of phot1 rapidly leads to a seedling-wide signal.

Taken together, the results presented in this study show that phototropic bending of etiolated *Arabidopsis* seedlings requires local expression and activation of phot1 in the upper hypocotyl, below the cotyledonary node, to perceive the phototropic stimulus and induce a signaling cascade that rapidly spreads to all cell layers throughout the hypocotyl. Thus, in etiolated seedlings spatial aspects of phototropism differ between monocots and dicots. Interestingly, the morphological and/or physiological adaptations occurring upon de-etiolation may alter these spatial effects on hypocotyl phototropism (Figures 1 and S1).

Supplemental Information

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at...

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Figure legends

Figure 1. Cotyledons and hypocotyl apex are dispensable for phototropism

(A) Phototropic bending of decapitated wild-type *Arabidopsis* (ecotype Col-0) seedlings. The large image on the left shows the upper hypocotyl of a three-day-old, etiolated seedling. Sites of decapitation (positions 1-3) are indicated with black lines and numbers. Seedlings were cut at indicated positions prior to irradiation with unilateral blue light at 0.1 µmol m^{-2} s^{-1}. An exemplary seedling for each cutting position after six hours of illumination is shown (small images). The number of seedlings showing phototropic bending, in relation to the total number of seedlings observed, is displayed on the right. Note that amputated upper parts of seedlings cut at position 3 respond to the light stimulus (numbers in brackets). The arrow indicates the direction of blue light.

(B) Elongation growth rates of untreated and decapitated seedlings during the experiment described in (A). Length of hypocotyls was measured before and after six hours of unilateral irradiation. Positions 1 and 2 refer to the sites of decapitation described in (A). Data shown are means +/- (2 x standard error (SE)). Asterisks indicate a significant difference between triple mutants and controls (Student's t-test; $p < 0.01$).

(C) Phototropic bending of *pidwag1wag2* mutants. Three-day-old, etiolated seedlings grown on vertical plates with half-strength MS agar medium were irradiated with unilateral blue light at a fluence rate of 0.1 µmol m^{-2} s^{-1}. Curvature was determined at the indicated times after the start of irradiation. A segregating *pid$^{+/-}$wag1wag2* line was used and control seedlings with normal cotyledons were analyzed in the same experiment. Data shown are means +/- (2 x SE) of 16 (triple mutant) and 48 (control) seedlings, respectively. Asterisks

indicate a significant difference between triple mutants and controls (Student's t-test; ** $p < 0.01$; * $p < 0.05$). BL, blue light.

(D) Elongation growth rate of *pidwag1wag2* mutants was determined using the same seedlings as in (C). Length of hypocotyls was measured before and after 24 hours of irradiation. Data shown are means +/- (2 x SE). Asterisks indicate a significant difference between triple mutants and controls (Student's t-test; $p < 0.01$).

See also Figure S1.

Figure 2. Expression of phot1-mCitrine in upper aerial parts rescues phototropism

(A) Exemplary upper hypocotyl of an etiolated *Arabidopsis* seedling and schematic representation of utilized promoter constructs. Expected expression domains are pointed out by black arrows.

(B) Phototropic bending of transgenic *Arabidopsis* seedlings expressing phot1-mCitrine from organ-specific promoters. Three-day-old, etiolated seedlings grown on vertical plates with half-strength MS agar medium were irradiated with unilateral blue light at a fluence rate of 1 μmol m^{-2} s^{-1}. Curvature was determined at the indicated times after the start of irradiation. The wild-type (Col-0), pPHOT1, and *phot1phot2* seedlings were analyzed in the same experiment. Data shown are means +/- (2 x SE) of 57-137 seedlings per genotype. BL, blue light.

See also Figure S2.

Figure 3. Expression of phot1-mCitrine in any cell layer restores phototropism

(A) Schematic representation of a hypocotyl cross section of an *Arabidopsis* seedling. Epidermis (red), cortex (blue), and endodermis (black) are shown and expected expression domains of utilized promoters are depicted.

(B) Phototropic bending of transgenic *Arabidopsis* seedlings expressing phot1-mCitrine from tissue-specific promoters. Seedlings were grown and treated as described in Figure 2B. Curvature was determined at the indicated times after the start of irradiation. Data shown in Figure 2B and Figure 3B were obtained in the same experiment but are presented in separate graphs for clarity; thus the measurements shown for pPHOT1 and *phot1phot2* control seedlings are identical in both graphs. Data shown are means +/- (2 x SE) of 57-143 seedlings per genotype. BL, blue light.

See also Figure S3.

Figure 4. Local phot1 activation triggers a rapid seedling-wide NPH3 dephosphorylation

Three-day-old, etiolated seedlings grown on plates with half-strength MS agar medium were irradiated with blue light from the top at a fluence rate of 15 µmol m^{-2} s^{-1}. Samples were harvested at the indicated times after the start of irradiation. NPH3 protein was detected by Western blotting using an anti-NPH3 antibody. Schematic representations of hypocotyl cross-sections indicating the overlapping expression domains of NPH3 and phot1-mCitrine in pPHOT1 and pSCR, respectively, are shown above the blot. Ep, epidermis; C1 and C2, cortex layer 1 and 2, respectively; En, endodermis; BL, blue light.

See also Figure S4.

Figure 1
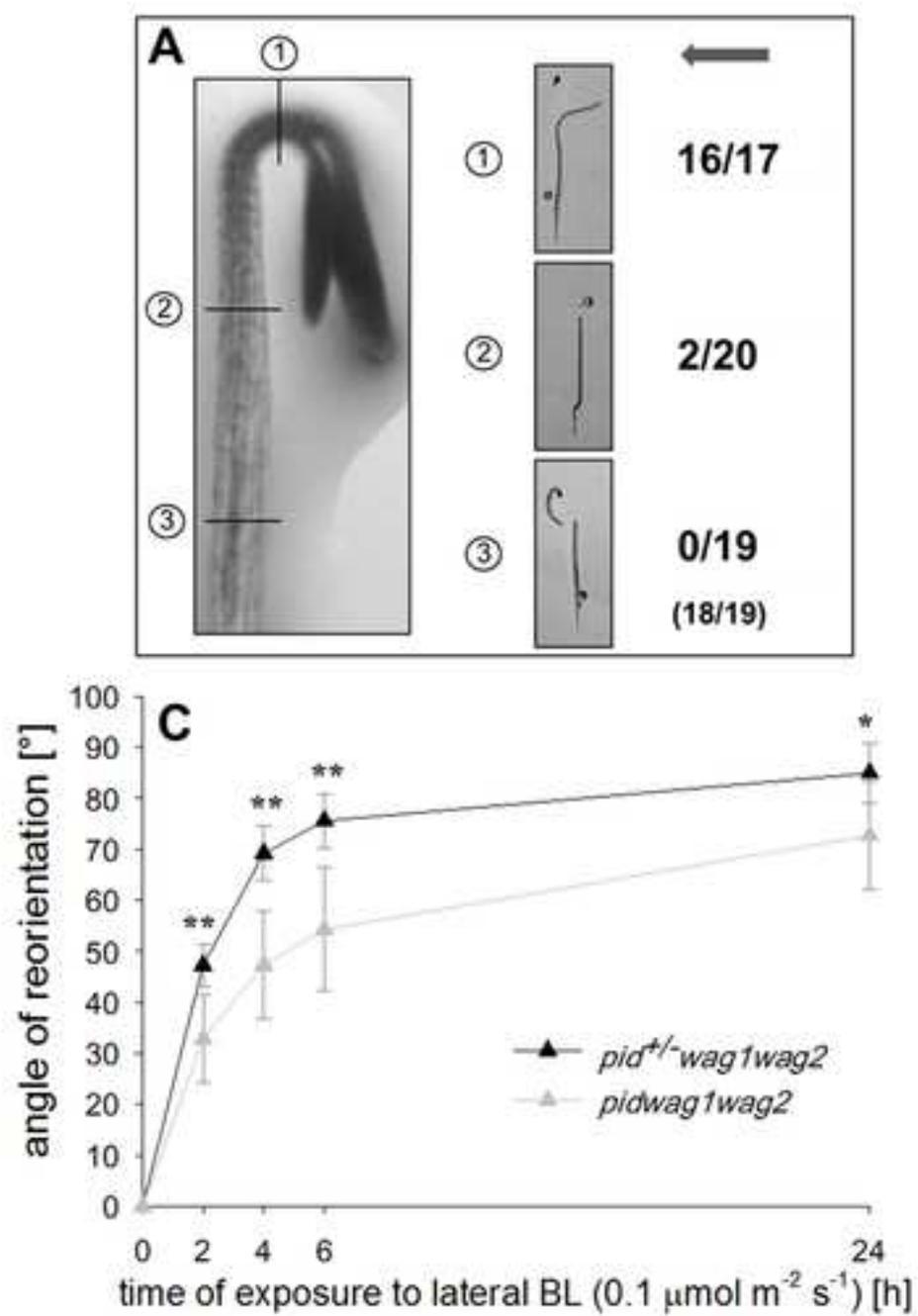
Figure 2
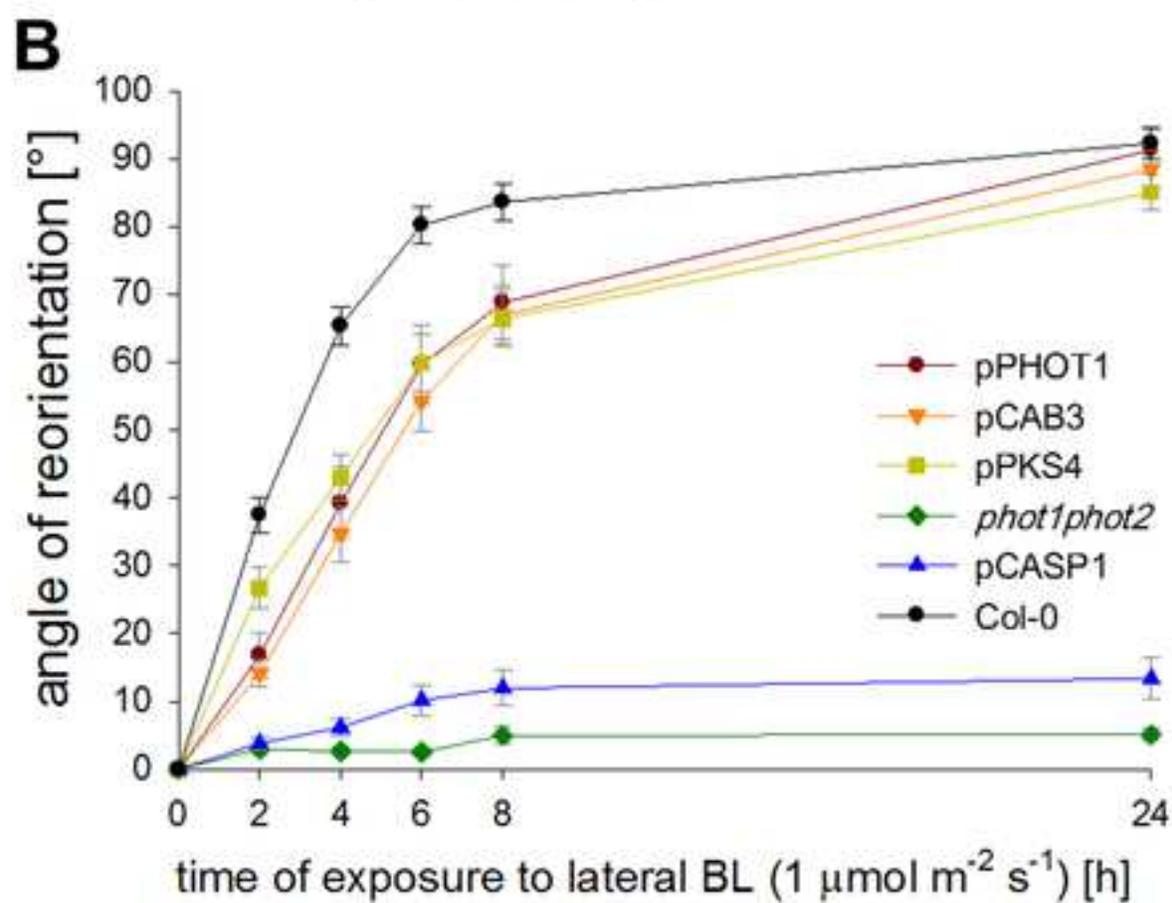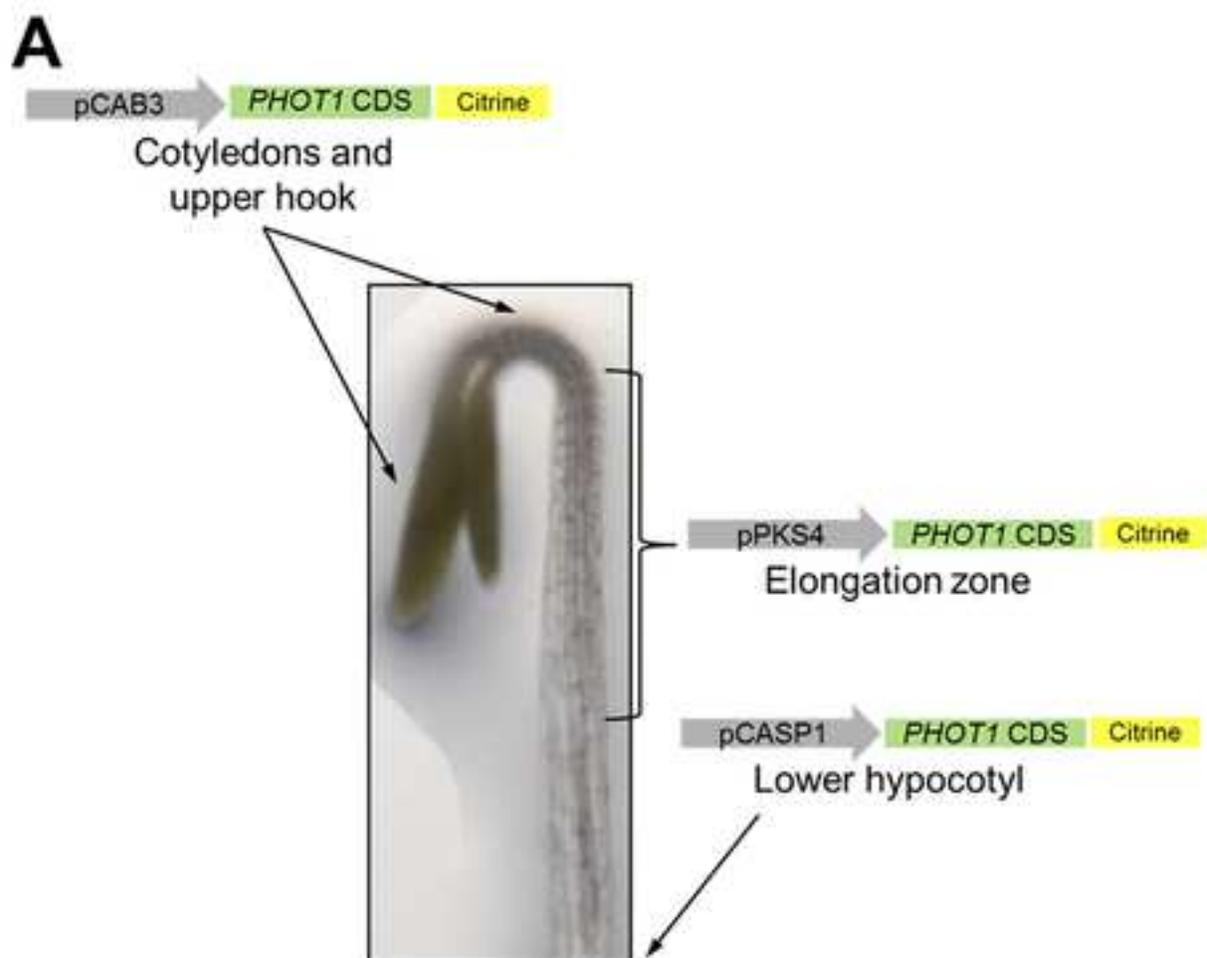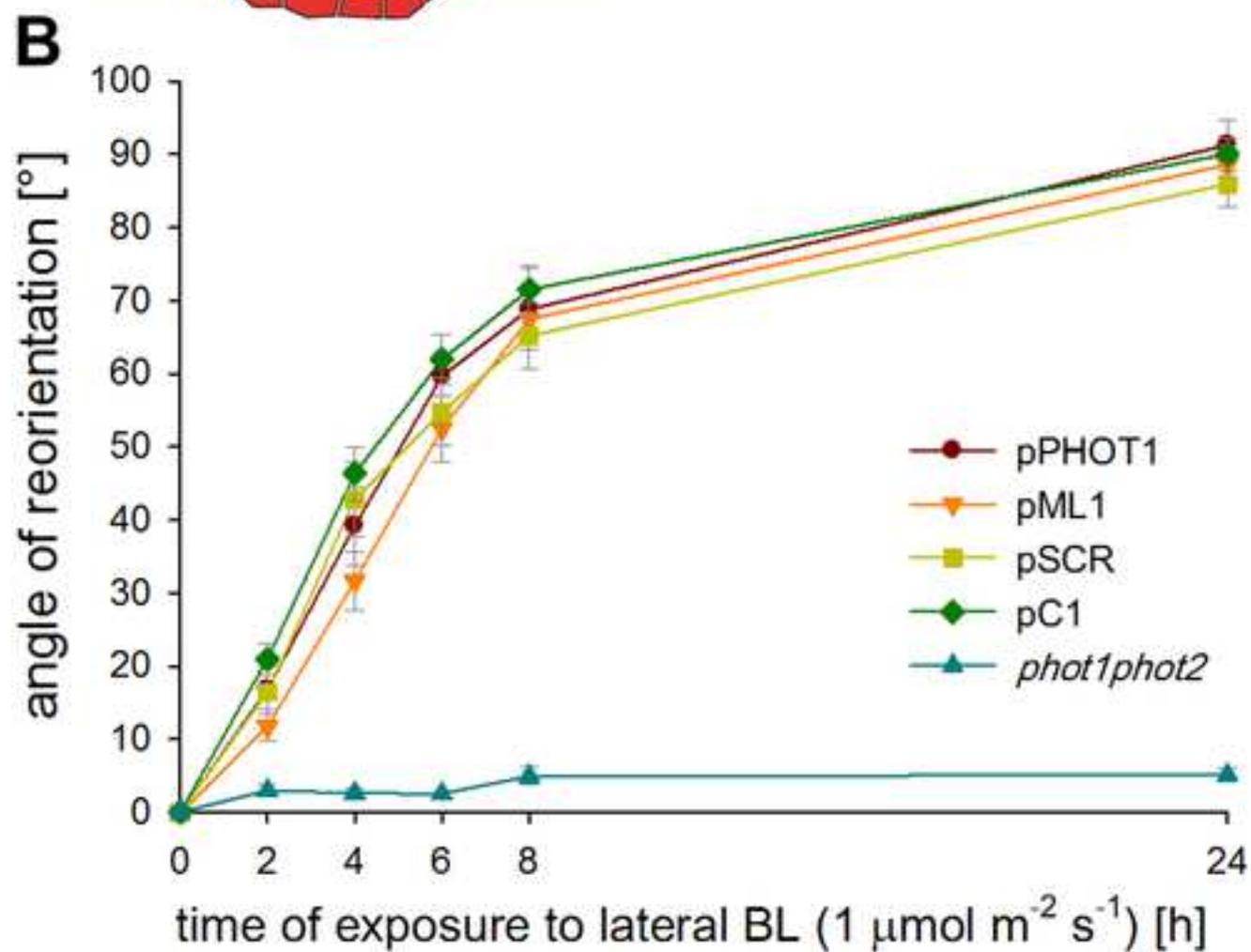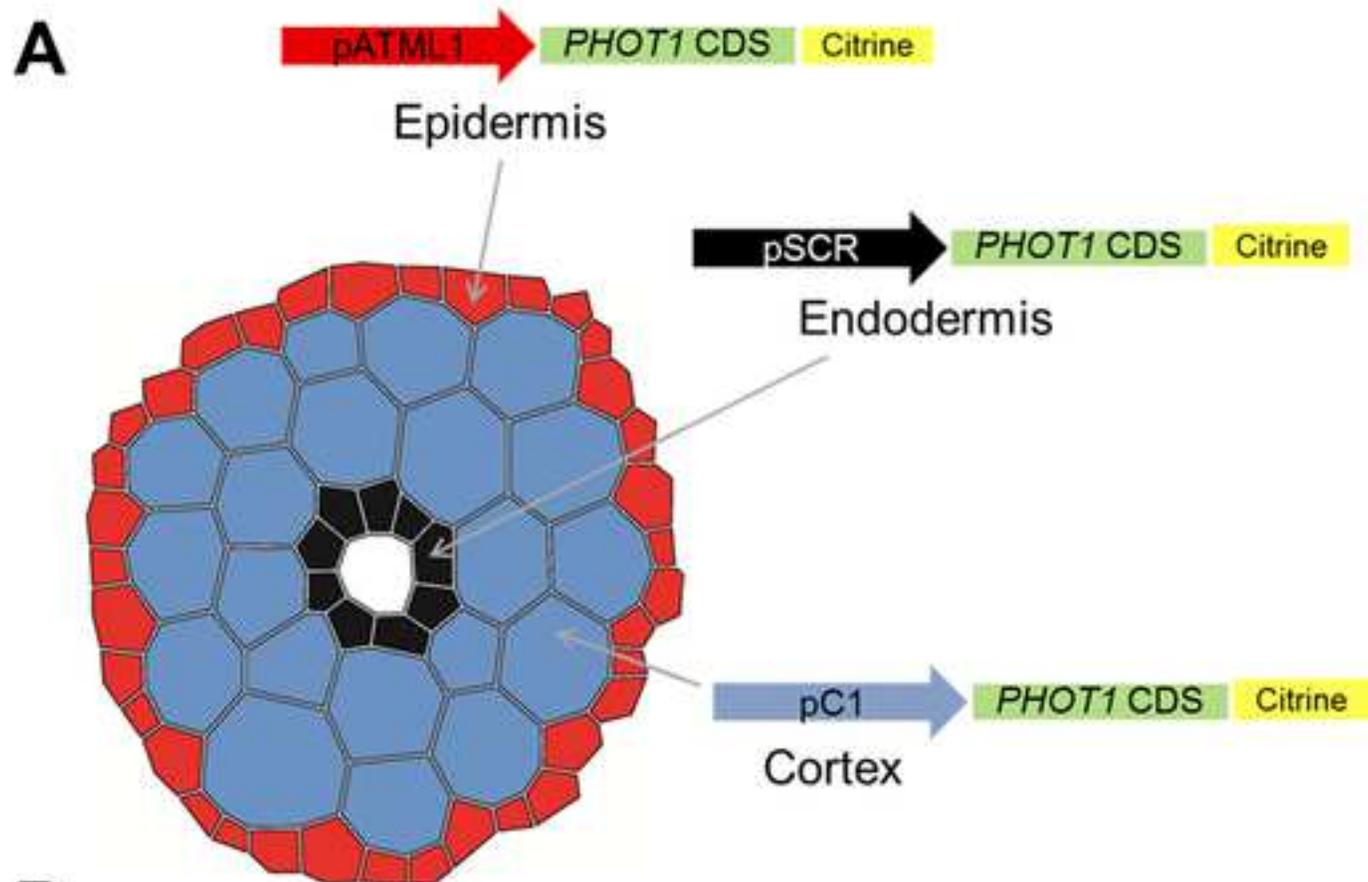
Figure 2
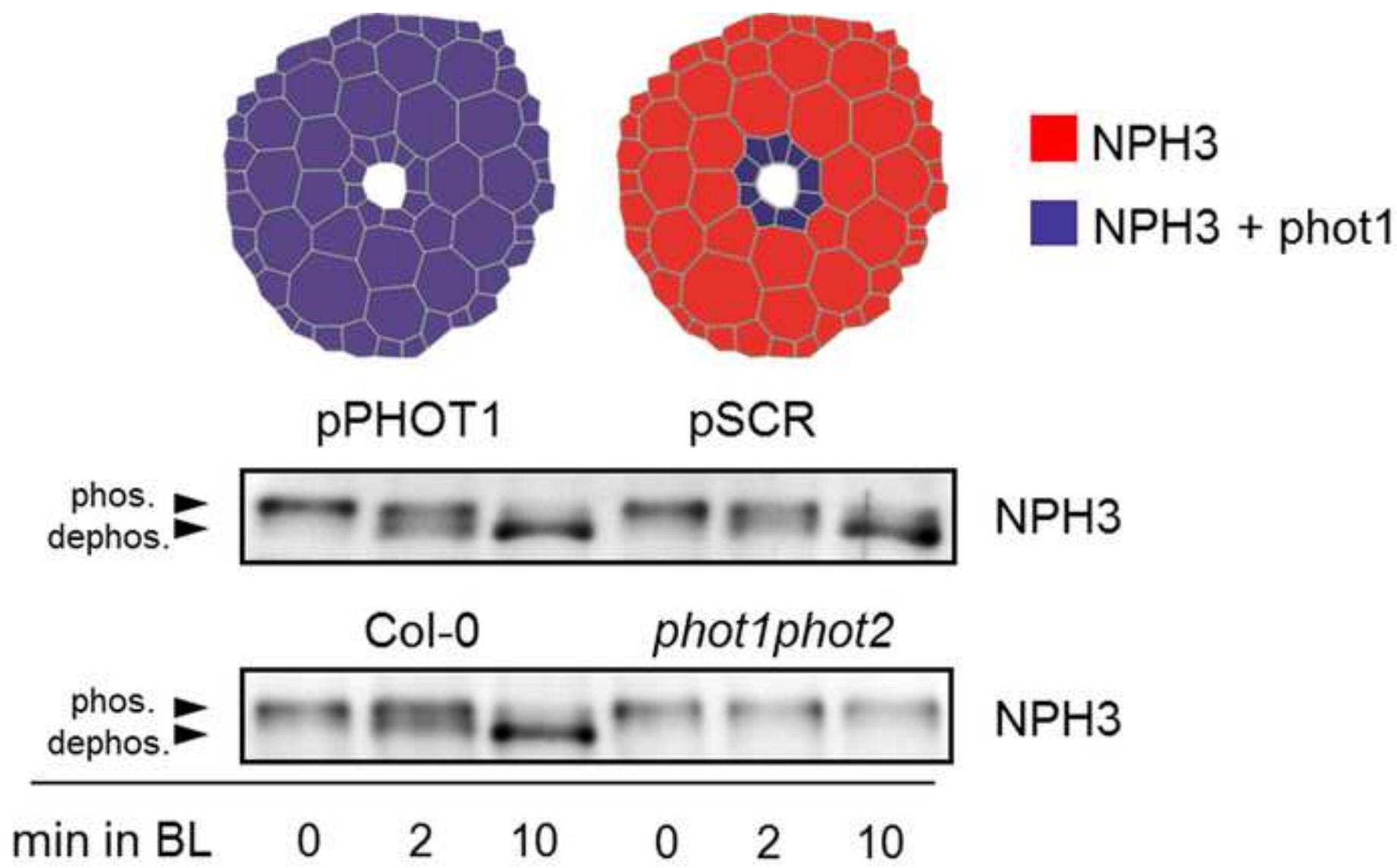
Figure 3

Figure 4

Defining the site of light perception and initiation of phototropism in *Arabidopsis.*

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Inventory of Supplemental Information

- **Supplemental Figure S1**. Phototropic bending of decapitated seedlings and *pidwag1wag2* triple mutants, related to Figure 1

- **Supplemental Figure S2**. Low levels of phot1-mCitrine are sufficient for normal phototropism, related to Figure 2

- **Supplemental Figure S3.** Expression patterns of phot1-mCitrine in transgenic lines, related to Figure 3

- **Supplemental Figure S4.** Dephosphorylation of NPH3 in blue light, related to Figure 4

- **Supplemental Experimental Procedures**

- **Supplemental References**
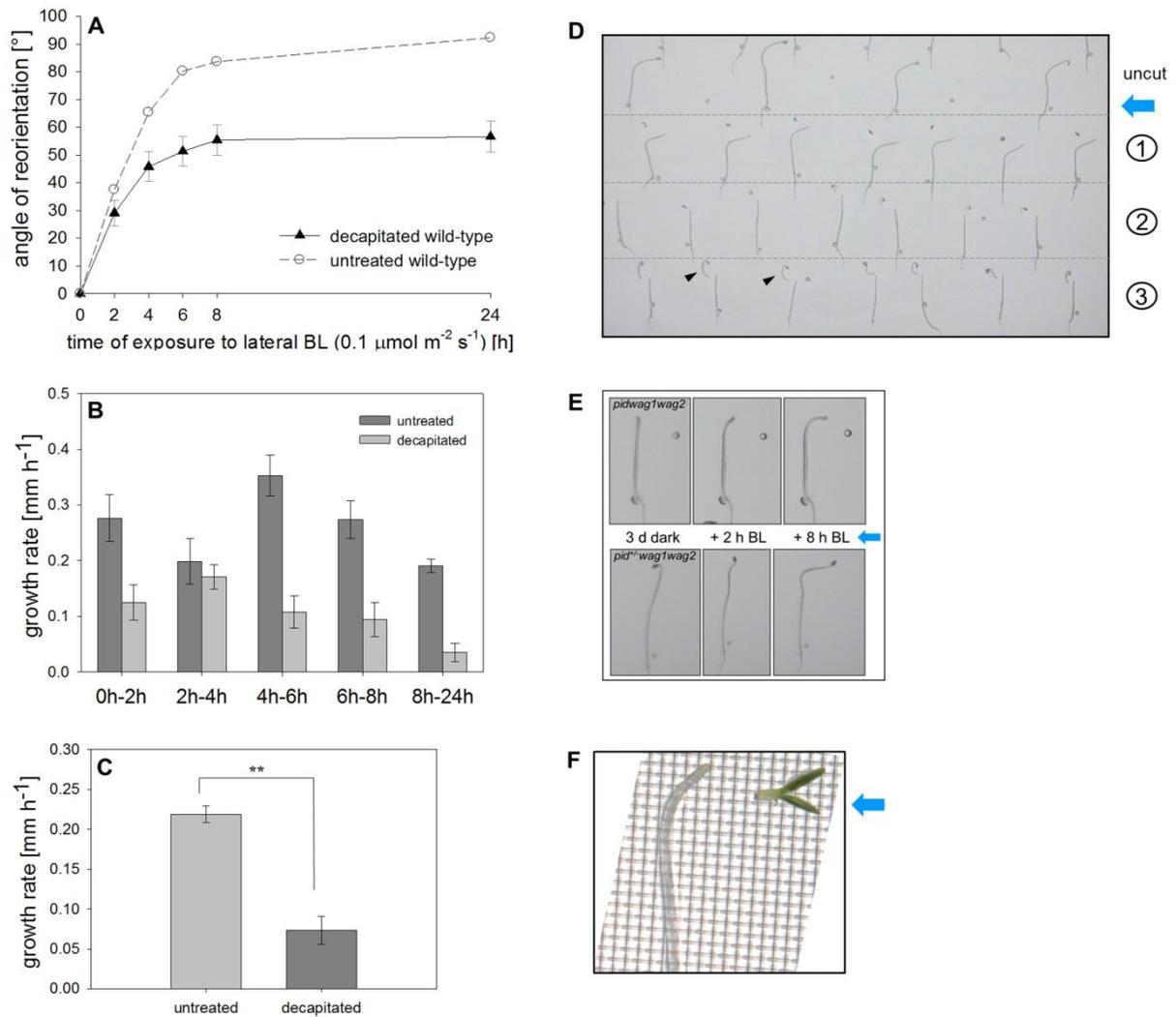
Figure S1. Phototropic bending of decapitated seedlings and *pidwag1wag2* triple mutants (Related to Figure 1)

(A) Phototropic bending of wild-type seedlings that were decapitated (position 1, see Fig. 1A) 90 minutes before irradiation with unilateral blue light at 0.1 μmol m^{-2} s^{-1}. Curvature was determined at the indicated times after the start of irradiation. Data shown are means +/- (2 x SE) of 28 seedlings. Bending characteristics of untreated seedlings (measured in a separate experiment, see Fig. S2A) are shown for comparison (dashed line). BL, blue light.

(B) Progression of elongation in untreated and decapitated wild-type seedlings. Elongation growth rates were determined using the same seedlings as in (A). Length of

hypocotyls was measured between the indicated time points after the start of unilateral irradiation. Data shown are means +/- (2 x SE).

(C) Elongation growth rate of untreated and decapitated seedlings during 24 hours. Length of hypocotyls was measured before and after 24 hours of unilateral irradiation. Data shown are means +/- (2 x SE). Asterisks indicate a significant difference between decapitated and untreated seedlings (Student's t-test; ** $p < 0.01$).

(D) Three-day-old, etiolated *Arabidopsis* seedlings (Col-0) grown and decapitated as described in Figure 1 prior to unilateral irradiation with blue light (0.1 μmol m^{-2} s^{-1}) for nine hours. Untreated seedlings are shown as control (upper row). Row numbers 1 to 3 refer to cutting positions as described in Figure 1. The arrow indicates the direction of blue light. Note the bending of amputated upper parts of hypocotyls in row 3 (arrowheads).

(E) Three-day-old, etiolated *pidwag1wag2* seedlings grown on vertical plates with half-strength MS agar medium were irradiated with unilateral blue light (0.1 μmol m^{-2} s^{-1}). Photographs were taken at the indicated times after the start of irradiation. A segregating *pid$^{+/-}$wag1wag2* population was used for the experiment. A homozygous triple mutant seedling is shown in the upper row; the lower row shows a seedling with normal cotyledons from the same plate. The arrow indicates the direction of blue light. BL, blue light.

(F) Phototropic bending of a de-etiolated wild-type (Col-0) seedling that has been decapitated prior to irradiation with unilateral blue light (1 μmol m^{-2} s^{-1}). Cotyledons, including the cotyledonary node and the apical meristem have been removed. The photograph was taken 24 hours after the start of irradiation. The arrow indicates the direction of blue light.
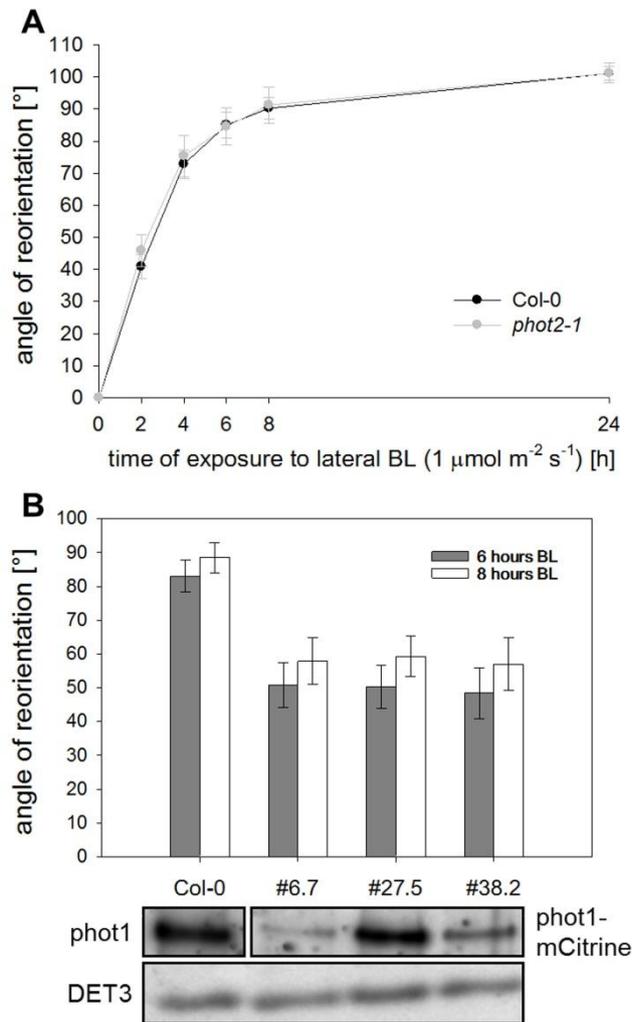
Figure S2. Low levels of phot1-mCitrine are sufficient for normal phototropism (Related to Figure 2)

(A) Phototropic curvature in our conditions is not influenced by phot2. Seedlings were treated as in Figure 2B and bending was measured at the indicated time points. Data shown are means +/- (2 x SE) of 76-84 seedlings per genotype. BL, blue light.

(B) Phototropic curvature of three-day-old, etiolated seedlings grown as in Figure S1. Curvature was determined after six and eight hours of unilateral irradiation. Three independent pPHOT1 lines were compared to the wild-type (ecotype Col-0). Line #38.2 is the one used in Figures 2B and 3B. Data shown are means +/- (2 x SE) of 54-132 seedlings per genotype. Protein expression levels of phot1 (Col-0) and phot1-mCitrine (pPHOT1 lines) were determined by Western blotting with specific antibodies against

phot1 using crude protein extracts of three-day-old etiolated seedlings. DET3 protein levels served as loading control. BL, blue light.
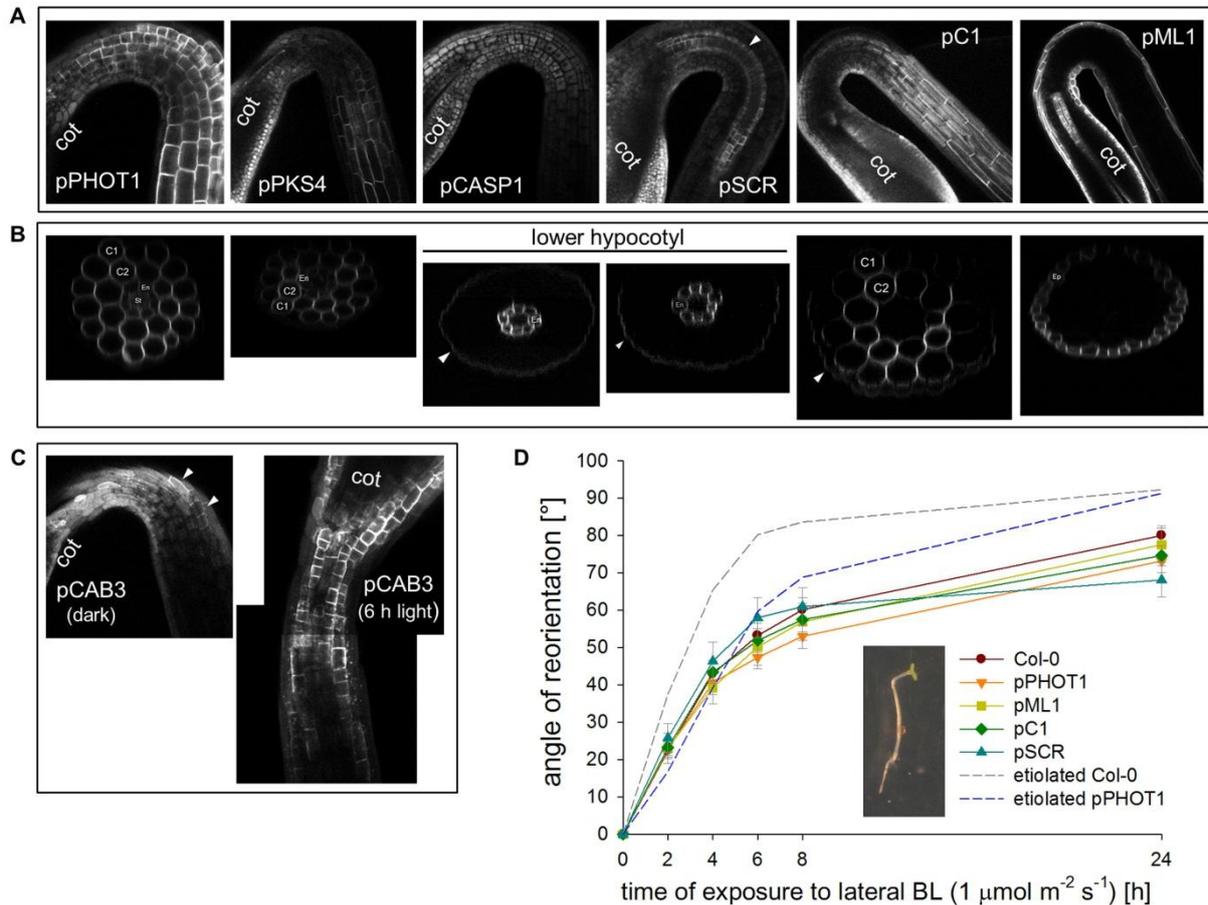
Figure S3. Expression patterns of phot1-mCitrine in transgenic lines (Related to Figure 3)

(A) Plasma membrane-localized phot1-mCitrine, expressed from the indicated promoters in the upper hypocotyl region of etiolated seedlings, was detected by 2-photon-microspcopy. Note phot1-mCitrine expression in the apical hook of pSCR (white arrowhead) and absence of phot1-Citrine signal in pCASP1. Signal detected inside the cells and not at the plasma membrane (e.g. in the hook and cotyledons of pCASP1) represents autofluorescence. cot, cotyledons.

(B) Expression of phot1-mCitrine in etiolated hypocotyls (plasma membrane-localized). Images were taken with a 2-photon microscope and reconstructed hypocotyl cross

sections from different promoter lines are shown (order as in (A)). White arrowheads indicate auto fluorescence from the cuticle. Ep, epidermis; C1 and C2, cortex layers 1 and 2, respectively; En, endodermis.

(C) Expression of phot1-mCitrine in pCAB3 (plasma membrane-localized). As expression from pCAB3 is strongly induced by light, the expression pattern after six hours of irradiation with white light is additionally shown. White arrowheads mark cells expressing phot1-mCitrine in the hook of an etiolated seedling. Signal detected inside the cells and not at the plasma membrane represents autofluorescence. cot, cotyledons. Note that although the plasma membrane-localized signal of phot1-mCitrine is easily detectable, we cannot ultimately exclude that very small and with our methods undetectable amounts of phot1-mCitrine are expressed in other tissues.

(D) Phototropic bending of de-etiolated transgenic *Arabidopsis* seedlings expressing phot1-mCitrine from tissue-specific promoters. Four-day-old, de-etiolated seedlings grown on vertical plates with half-strength MS agar medium were irradiated with unilateral blue light at a fluence rate of 1 µmol m^{-2} s^{-1}. Curvature was determined at the indicated times after the start of irradiation. Data shown are means +/- (2 x SE) of 47-92 seedlings per genotype. Dashed lines indicate bending rates of etiolated wild-type (grey line) and pPHOT1 (blue line) seedlings from the experiment described in Fig. 2B for comparison. The small inlet shows an exemplary de-etiolated wild-type seedling after 24 hours of irradiation. BL, blue light.
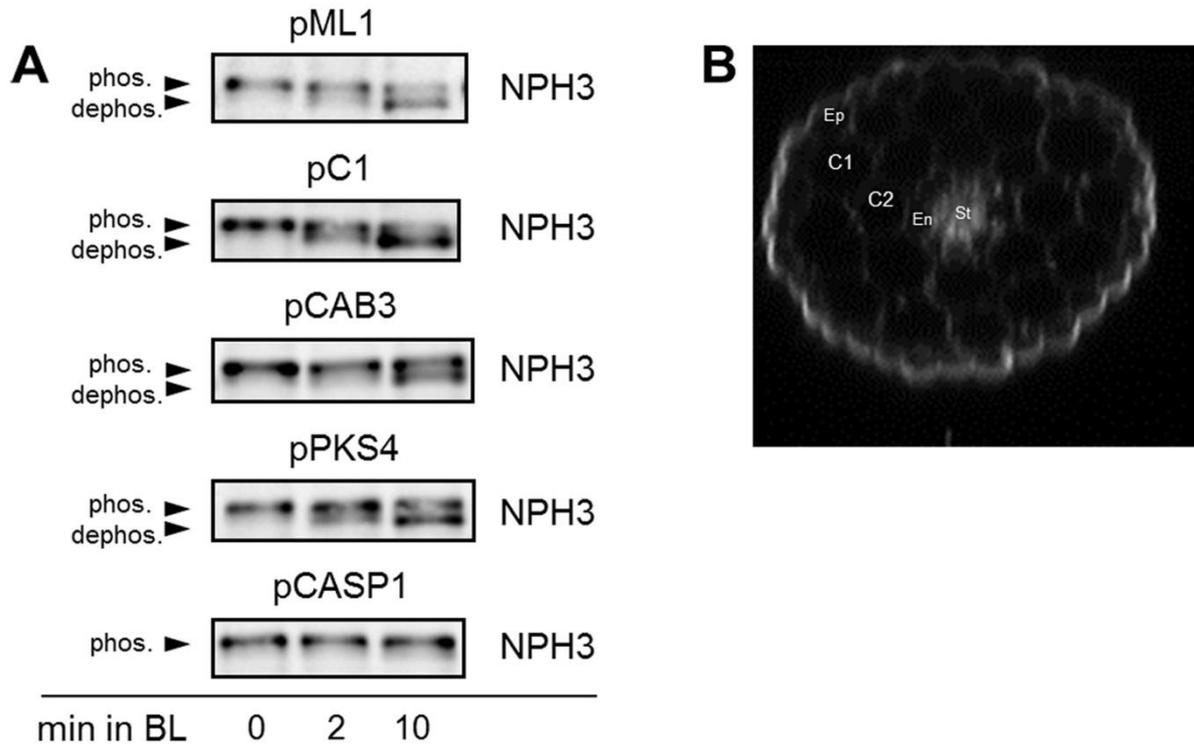
Figure S4. Dephosphorylation of NPH3 in blue light (Related to Figure 4)

(A) Three-day-old, etiolated seedlings grown on plates with half-strength MS agar medium were irradiated with blue light from the top at a fluence rate of 15 μmol m^{-2} s^{-1}. Samples were harvested at the indicated times after the start of irradiation. NPH3 protein was detected by Western blotting using an anti-NPH3 antibody. BL, blue light.

(B) Expression domain of NPH3 in etiolated seedlings. Transgenic seedlings expressing NPH3-mCherry from its own promoter (*nph3-6, NPH3::NPH3-mCherry*) were grown as in (A) and analyzed by 2-photon microscopy. A cross section of the upper hypocotyl was reconstructed using the ImageJ software. Ep, epidermis; C1 and C2, cortex layer 1 and 2, respectively; En, endodermis; St, stele.

Supplemental Experimental Procedures

Plant material and growth conditions

The Columbia (Col-0) ecotype of *Arabidopsis thaliana* was used as the wild-type and all used mutant alleles were in the Col-0 background. The following mutants have been described previously: *pid+/-wag1wag2* [1], *phot2-1* [2], and *phot1-5phot2-1* [3]. The transgenic *nph3-6, NPH3::NPH3-mCherry* line was a gift from Ullas Pedmale and Emmanuel Liscum. Unless stated otherwise, seeds were surface-sterilized (three minutes in 70 % (v/v) ethanol + 0.05 % (v/v) Triton X-100, then ten minutes in 100 % (v/v) ethanol) plated on half-strength Murashige and Skoog medium with 0.8 % (w/v) agar and kept at 4 °C in the dark for three days. Plates were then transferred to 21 ± 1 °C (Sanyo incubator) and exposed to 50 µmol m^{-2} s^{-1} red light for 2-3 hours to induce germination prior to incubation in the dark at 21 ± 1 °C (Heraeus incubator) for 65 – 70 hours. The red light source was a light-emitting diode (λmax, 664 nm; CLF Plant Climatics GmbH). Light intensities were determined with an International Light IL1400A photometer (Newburyport, MA) equipped with an SEL033 probe with appropriate light filters.

NPH3-dephosphorylation assays

Three-day-old, etiolated *Arabidopsis* seedlings were obtained as described and exposed to blue light from above at an intensity of 15 µmol m^{-2} s^{-1} for the indicated times. 50 seeds were sown for each time point. The blue light source was a light-emitting diode (λmax, 462 nm; CLF Plant Climatics GmbH). Total proteins were extracted at the indicated time points by grinding the seedlings in 100 µl 2x Laemmli buffer [0.125 M Tris pH 6.8; 4 % (w/v) SDS; 20 % (v/v) glycerol; 0.02 % (w/v) bromophenol blue; 10 % (v/v) β-mercaptoethanol].

Measurement of hypocotyl curvature and growth rates

For phototropism experiments, seedlings were grown on vertically orientated plates for three days in darkness at 21 ± 1 °C before irradiation with unilateral blue light at 0.1 or 1 μmol m^{-2} s^{-1} as indicated. When de-etiolated seedlings were used, they were grown on vertically orientated plates for three days in darkness at 21±1 °C and then irradiated with 150 μmol m^{-2} s^{-1} white light for eight hours so that the apical was opened and the cotyledons expanded and green. Subsequently they were kept in darkness for 16 hours before irradiation with unilateral blue light as indicated. The blue light source was a light emitting diode (λmax, 462 nm; CLF Plant Climatics GmbH). Plates were photographed in infrared light at the indicated times. Hypocotyl angles relative to growth direction before the start of blue light irradiation were measured using National Institutes of Health ImageJ software version 1.45s (http://rsb.info.nih.gov/ij). Growth rates were determined by measuring hypocotyl length of the same seedlings used for phototropism measurements before and after 24 hours of irradiation using the ImageJ software.

Decapitation experiments

For decapitation experiments, seeds were sown on nylon mesh (160 mm, Micropore) disposed on the surface of plates with half-strength Murashige and Skoog medium with 0.8 % (w/v) agar. Three-day-old etiolated or four-day-old de-etiolated *Arabidopsis* seedlings (Col-0) were obtained as described above and cut at indicated positions using micro scissors (Hammacher, Solingen, Germany) prior to phototropism assays. Manipulations were carried out in a dark room under dim green light.

Plasmid construction and transgenic lines

Standard cloning procedures were used for plasmid construction. Plant transformation vectors were based on pGREENII series (http://www.pgreen.ac.uk). The basis vector (JA019) for all used constructs was a gift from Julien Alassimone and Niko Geldner and contained the *SCR* promoter region [4] and the coding sequence of mCitrine [5]. The full length coding sequence of phot1 [6] was inserted into this vector so that the *PHOT1* gene was fused translationally with the *mCitrine* gene to give *pSCR::PHOT1-mCitrine*. To obtain other constructs, the *SCR* promoter was replaced by the respective promoters that are described in the following. The *ML1* promoter [7] was amplified from the *pMLBART/ ML1-bes1-D–GFP* vector, a gift from Sigal Savaldi-Goldstein and Joanne Chory. The *CAB3* promoter [8] was amplified from BAC F1N18 (obtained from ABRC). The *PKS4* promoter (1.5 kb upstream of the initiation codon) was amplified from BAC F21E1 (ABRC). The *AT1G09750* (*C1*) promoter (1.7 kb upstream of the initiation codon) [9] was amplified from *pDONR-P4-P1R-pC1*, a gift from Miguel Blázquez. The *CASP1* promoter [10] was provided by Joop Vermeer. The *PHOT1* endogenous promoter was obtained by amplifying 3.9 kb upstream of the initiation codon from the LEGT2 transformation vector [6]. Transgenic plants were generated by introduction of the plant expression constructs into a pSOUP containing *Agrobacterium tumefaciens* strain GV3101. Transformation was done by floral dipping [11]. Plasmids contained Basta resistance for plant selection. Based on segregation of Basta-resistance, homozygous T_3 lines with a single transgene locus were selected.

SDS-PAGE and immunoblot analysis

Total proteins were separated on 8 % SDS-PAGE gels and transferred onto nitrocellulose membranes with 100 mM Tris-Glycin buffer [25 mM Tris, 192 mM glycine] + 10% (v/v)

ethanol. The blots were probed with anti-DET3, anti-phot1, and anti-NPH3 antibodies as described in [12]. Chemiluminescence signals were generated using Immobilon Western HRP Substrate (Millipore). Signals were captured with a Fujifilm ImageQuant LAS 4000 mini CCD camera system and quantifications were performed with ImageQuant TL software (GE Healthcare).

Microscopy

Images of mCitrine-fluorescence in transgenic lines were taken with a 2-photon Zeiss LSM 710 NLO microscope equipped with a Chameleon Ultra II Ti:Sapphire infrared laser at 960 nm using the non-descanned detector (NDD) with a 500–550 bandpath filter and a W Plan-Apochromat 20x lens. Cross-sections were reconstructed from z-stacks using the ImageJ software.

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