

The Subcellular Localization and Blue-Light-Induced Movement of Phototropin 1-GFP in Etiolated Seedlings of *Arabidopsis thaliana*^W

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ABSTRACT Phototropin 1 (*phot1*) is a photoreceptor for phototropism, chloroplast movement, stomatal opening, leaf expansion, and solar tracking in response to blue light. Following earlier work with *PHOT1::GFP* (Sakamoto and Briggs, 2002), we investigated the pattern of cellular and subcellular localization of *phot1* in 3–4 d old etiolated seedlings of *Arabidopsis thaliana*. As expressed from native upstream sequences, the *PHOT1::GFP* fusion protein is expressed strongly in the abaxial tissues of the cotyledons and in the elongating regions of the hypocotyl. It is moderately expressed in the shoot/root transition zone and in cells near the root apex. A fluorescence signal is undetectable in the root epidermis, root cap, and root apical meristem itself. The plasma membranes of mesophyll cells near the cotyledon margin appear labeled uniformly but cross-walls created by recent cell divisions are more strongly labeled. The pattern of labeling of individual cell types varies with cell type and developmental stage. Blue-light treatment causes *PHOT1::GFP*, initially relatively evenly distributed at the plasma membrane, to become reorganized into a distinct mosaic with strongly labeled punctate areas and other areas completely devoid of fluorescence—a phenomenon best observed in cortical cells in the hypocotyl elongation region. Concomitant with or following this reorganization, *PHOT1::GFP* moves into the cytoplasm in all cell types investigated except for guard cells. It disappears from the cytoplasm by an unidentified mechanism after several hours in darkness. Neither its appearance in the cytoplasm nor its eventual disappearance in darkness is prevented by the translation inhibitor cycloheximide, although the latter process is retarded. We hypothesize that blue-light-induced *phot1* relocalization modulates blue-light-activated signal transduction.

INTRODUCTION

To optimize growth and photosynthesis, plants have evolved elaborate mechanisms to sense and respond to light signals from the environment. One of these mechanisms is phototropism. In general, stems and stem-like organs of flowering plants are positively phototropic; they bend toward a light source, thus optimizing photon capture for photosynthesis. Negative phototropic responses can operate to prevent damage caused by excess light. Under stress, some leaves will fold or rotate to reduce photon capture (Koller, 2000). Typically, plant roots are negatively phototropic to blue light but respond positively to red light (Kiss et al., 2003). *Arabidopsis thaliana* exhibits this pattern of shoot and root phototropism. Blue-light-induced phototropism in this model species is mediated by the phototropins, *phot1* and *phot2* (Briggs and Christie, 2002).

The phototropins are also photoreceptors for blue-light-induced chloroplast movements, stomatal opening, leaf ex-

pansion, rapid inhibition of hypocotyl growth (Christie, 2007), and solar tracking (Inoue et al., 2005, 2007). These are all responses that serve to maximize growth potential under favorable conditions and minimize damage under a range of stress conditions. Details of the down-stream signal-transduction pathways remain obscure and the phototropins appear to use different pathways for different light responses. For example, *NPH3*—a protein that interacts directly with *phot1* (Motchoulski and Liscum, 1999)—is required for phototropism but not for stomatal opening or chloroplast relocation. *RPT2* (a member of the same protein

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family as NPH3) is required both for phototropism and stomatal opening, but not for chloroplast relocation (Inada et al., 2004). In addition, cytoplasmic concentrations of Ca^{2+} , K^+ , Na^+ , Cl^- , and H^+ can change during phototropin-mediated blue-light signaling, indicating that changes in some ion channels and/or pumps are mediated by phototropins (Babourina et al., 2003; Baum et al., 1999; Fuchs et al., 2003; Harada et al., 2003; Stoelzle et al., 2003). Recent studies indicate that *phot1* plays a role in drought tolerance in *Arabidopsis* (Galen et al., 2007).

According to the classic Cholodny-Went theory of tropisms, asymmetric redistribution of auxin in response to gravity or unilateral light leads to organ curvature by causing unequal rates of cell elongation (Went and Thimann, 1937). This theory is supported by recent studies on auxin transport and its role in tropisms (Friml et al., 2002) and the patterning of cell types (Blilou et al., 2005). The putative auxin polar transport facilitators in *Arabidopsis* (members of the PIN protein family) are polarly localized in the apical and/or basal plasma membrane (PM) to allow for polar release of auxin. These PIN proteins cycle between the PM and endocytic vesicles. Evidence for this cycling comes from work with inhibitors of auxin transport. For example, *N*-naphthylphthalamic acid (NPA) and 2,3,5 triiodobenzoic acid (TIBA), inhibitors of auxin transport, and brefeldin A (BFA) (an inhibitor of protein secretion) all block auxin transport by inhibiting the endocytosis of PIN proteins (Geldner et al., 2001, 2003). A member of the PIN family—PIN3—is reported to play an important role in the redistribution of auxin in the root cap under gravistimulation (Friml et al., 2002). Another member of the PIN family—PIN1—becomes re-localized in cells on the shaded side of the hypocotyl of wild-type *Arabidopsis* but is not similarly re-localized in a *phot1* mutant (Blakeslee et al., 2004). Thus, *phot1* excitation by blue light has an influence on the intracellular position of one of the proteins involved in auxin transport in the hypocotyl. Hence, PIN proteins might be involved in the phototropin signaling pathway for phototropism.

Sakamoto and Briggs (2002) used a *PHOT1::GFP* construct driven by the native *phot1* promoter, transformed into an *Arabidopsis phot1-5 (nph1-5)* null mutant, to investigate the cellular and subcellular localization of *phot1* in the absence or presence of blue light. They observed *PHOT1::GFP* expression in nearly all cell types, but the strongest expression was seen at the PM of dividing and elongating cells of root, shoot, and cotyledons, both in etiolated seedlings and in leaf epidermal cells, mesophyll cells, and guard cells in light-grown seedlings. The strong expression of *phot1* in these developmentally and physiologically active cells is consistent with the known physiological roles of *phot1*.

Sakamoto and Briggs (2002) also reported that a fraction of the *PHOT1::GFP* signal was released into the cytoplasm after blue-light illumination. This phenomenon was shown to occur in both cortical and epidermal cells from the hook region of the hypocotyl. A time-course series of images of epidermal cells indicated that the movement of the *PHOT1::GFP* into

the cytoplasm could be detected as early as 3 min after the initial blue-light exposure from the confocal microscope scanning laser. After 1 h of continuous blue light, a fraction of the *PHOT1::GFP* could be detected by immunoblotting in a soluble fraction. In a similar study, Kong et al. (2006) observed movement of GFP-labeled *phot2* into the cytoplasm of epidermal cells of dark-grown *Arabidopsis* seedlings, and mesophyll protoplasts and guard cells from light-grown seedlings on irradiation with blue light. The fluorescence appeared in punctate structures that co-localized with a Golgi marker.

Plant responses to blue light involve many different cell types (Christie, 2007). Epidermal cells are involved in leaf expansion, cortical and epidermal cells are involved in phototropic curvature, guard cells provide the mechanism for blue-light regulation of stomatal aperture, and mesophyll cells regulate chloroplast position, the latter in a cell-autonomous manner (Tlalka et al., 1999). The purpose of this paper is two-fold: first, to provide a detailed description of the cellular and subcellular distribution of *PHOT1::GFP* driven by the native *phot1* promoter; and, second, to examine the subcellular redistribution of *PHOT1::GFP* following blue-light excitation in the many different cell types involved in responses to blue light. The possible physiological consequences of blue-light-induced phototropin redistribution in the different cells and tissues are discussed.

RESULTS

Cellular and Subcellular Localization of *PHOT1::GFP*

Distribution in Etiolated Seedlings

We examined *PHOT1::GFP* distribution in whole 4 d old dark-grown seedlings by fluorescence microscopy. At low magnification the strongest signals were found to arise from the hook and the elongation region of the hypocotyl, and across the abaxial faces of the cotyledons (Figure 1A, cotyledon abaxial face view; Figure 2, cotyledon edge view). The fluorescence signal declined concomitantly with elongation of the hypocotyl and expansion of the cotyledons. Whether this decline is simply the consequence of dilution through cell enlargement or a reduction in gene expression is not resolved in this study. Surprisingly, a strong signal arose from the shoot-root transition region (Figure 1A). This strong signal could arise because the cells are not elongating and diluting the signal, or because *PHOT1::GFP* expression is higher in these cells, or both. Fluorescence declined sharply below this region, but became considerably stronger in the more apical tissues of the root. The average brightness of GFP signal detected in different tissues of 10 d old seedlings was measured and the results are shown in Figure 1B.

Distribution in the Cotyledons

At higher magnification, confocal images resolved major differences in the tissue and cellular distribution of *PHOT1::GFP* in various tissues and organs. The strong signal seen from the

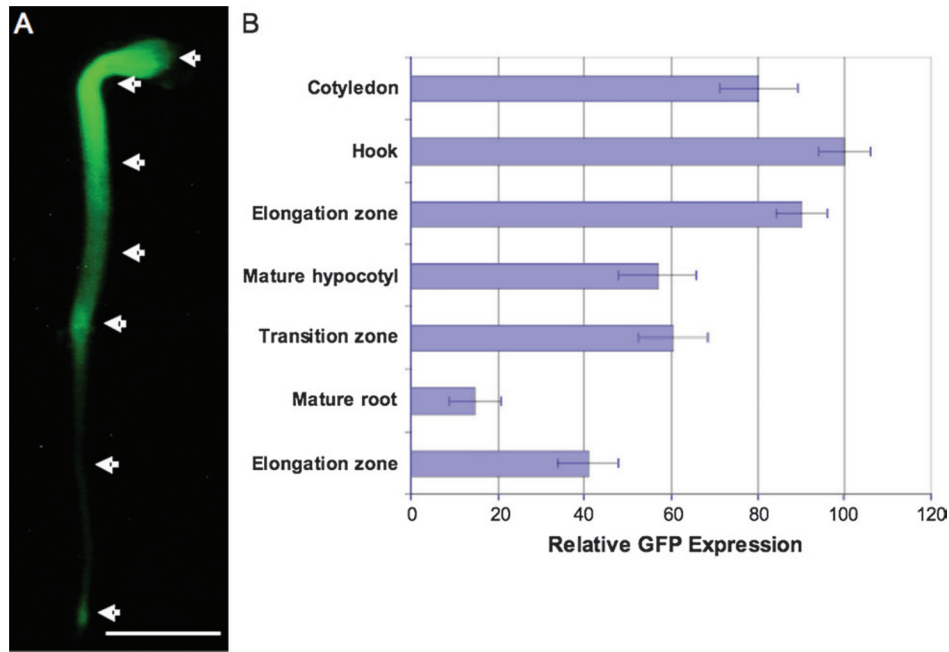


Figure 1. PHOT1::GFP Expression in Dark-Grown *Arabidopsis* Seedlings.

PHOT 1::GFP expression in a 4 d old *Arabidopsis* seedling.

(A) Image taken with a binocular low-power fluorescence microscope (Bar = 1 mm).

(B) Quantification of the intensities of the PHOT1::GFP fluorescence was obtained with ImageJ software. Arbitrary units, value for highest reading (hook) set to 100%. White arrows in Figure 1A indicate where measurements were taken. Averages from 10 seedlings. For details, see Materials and Methods. Error bars indicate standard error of the mean.

cotyledons in Figure 2 arises both from the epidermis on the abaxial face and the adjacent underlying mesophyll cells. It is almost undetectable from the adaxial tissues. As shown in Figure 3A, the signal from the epidermis at the margin of the cotyledon is extremely weak (thin arrows). However, the fluorescence from the underlying cell layers is far stronger, particularly on certain walls at right angles to the cotyledon surface (thick arrows, Figure 3A). These walls are cell plate-like and are likely the ones most recently laid down during the cell division that precedes cotyledon expansion. They are much more common near the cotyledon margin where the marginal meristem is located than toward the center.

The epidermal cells on the abaxial face of the cotyledon are heavily fluorescent (Figure 3A and 3B). However, the signal appears enriched at the anticlinal walls and weaker or undetectable from the inner and outer periclinal walls (Figure 3A, 3B and 3D). Using the reslice option of ImageJ from z-series scanning data, we were able to reconstruct transverse images from composite longitudinal images for Figures 3C, 3D and 5 (see below). Guard cells from 4 d old seedlings as visualized in median transverse section (Figure 3C) show signal predominantly on their anticlinal walls. (Note that the anticlinal walls in Figure 3C lie at different angles with respect to the vertical, making it unlikely that the intensity of their fluorescence is an artifact of their being oriented orthogonal to the optical axis

of scanning.) Individual epidermal cells thus resemble imaginative cookie cutters (Figure 3A and 3B). There is weak fluorescence at the walls of the mesophyll cells facing the epidermis (Figure 3A and 3B). In 4-d old seedlings, all of the anticlinal walls of both guard cells express PHOT1::GFP (Figure 3B). However, in seedlings 3 d old, incipient guard cells appear as dark circles with weak fluorescence apparent only around the periphery (Figure 3E, arrow, cf. Figure 3B, arrows). Thus, PHOT1::GFP expression is delayed in guard cells compared with its expression in adjacent epidermal cells.

Distribution in the Hook

As reported previously (Sakamoto and Briggs, 2002), epidermal cells in the apical hook region show lower expression than the underlying cortical cells (Figure 4A and 4B). The epidermal cells on the margins of the image show that as with the epidermal cells of the cotyledons, there is weaker signal at the outer periclinal walls (Figure 4A, arrows). In longitudinal section, it appears that it is the end walls of cortical cells and the wall adjacent to the epidermal cells that are most heavily labeled (Figure 4B, thick arrows). This distribution forms a 'C' in longitudinal section with the open side facing inward. The heavy signal marking the contact between the outer periclinal wall of the cortical cell and the inner periclinal wall of



Figure 2. Single Confocal Image Showing PHOT1::GFP Localization in the Cotyledon and Apical Hook Region.

PHOT1::GFP is preferentially localized at the epidermal anticlinal walls through the hook to the base of the cotyledon (see left side, arrows). The underlying adjacent cortical cells along the hook also show strong fluorescence at their outer periclinal walls adjacent to the epidermis and at their anticlinal walls, with little at their inner periclinal walls. Thus, each cell forms a 'C' shape. Only the abaxial cell layers of the cotyledon show strong PHOT1::GFP expression (Bar = 100 μm).

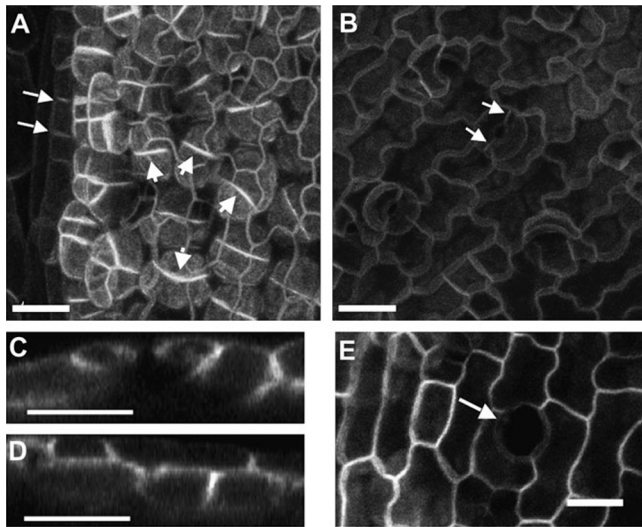


Figure 3. Distribution of PHOT1::GFP in the Cotyledons of 4 d Old Etiolated Seedlings.

(A) Brightest point projection of the abaxial face of a cotyledon near the cotyledon margin. The seedling was 4 d old. The image was constructed from a z-series of optical sections from the surface of the cotyledon to a depth of 30 μm . Epidermal cells at the left margin show only very weak PHOT1::GFP fluorescence, localized largely to the anticlinal walls in a polar fashion (long thin arrows). The outer faces of the mesophyll cells show fairly strong fluorescence at their plasma membranes in a somewhat mottled pattern, likely caused by the blue-light treatment from the laser while the sections were being imaged (see later). The epidermal anticlinal walls are uniformly labeled and there is almost no detectable signal from the outer or inner periclinal walls. Note the especially strong

the adjacent epidermal cell arises exclusively from the cortical cell (Figure 4B, dashed arrows).

Some PHOT1::GFP fluorescence also marks the deeper cells, likely pith and developing vascular tissue (Figure 4A and 4B) but, because of light scattering, the resolution is insufficient to provide more structural detail. Note that the end walls of these inner cells are generally more heavily labeled than the side walls (Figure 4B)—a polarity that occurs to varying degrees repeatedly throughout the seedling.

Changes from Hook to Transition Region as Visualized in Cross-Section

We reconstructed transverse images from stacks of longitudinal images for the four images in Figure 5A–5D, illustrating the distribution of PHOT1::GFP in sections from the hook (A), elongation zone (B), mature zone (C), and shoot–root transition region (D). The pattern of strong fluorescence at the anticlinal walls of the epidermal cells, noted above for the cotyledon epidermis, persists down the hypocotyl axis. In the hook, the first layer of cortical cells beneath the epidermis also shows the 'C'-shaped pattern with the opening facing in, just as was the case in longitudinal section (Figure 5A). Further down in the elongation zone, all of the longitudinal walls of the first row of cortical cells below the epidermis are labeled and the next layer in show the inward-facing 'C'-shaped distribution of

signal from cell plate-like structures in the center of the mesophyll cells (short thick arrows) (Bar = 20 μm).

(B) Brightest point projection of the abaxial face of a cotyledon near the cotyledon's center (4 d old seedling). The image was constructed from a z-series of images from the surface of the cotyledon to a depth of 30 μm . The oblique angle of observation shows the strong signal on the anticlinal walls of the epidermal cells and the lack of signal at inner or outer walls. The signal is fairly weak in underlying mesophyll cells except for an occasional strongly fluorescent cell plate-like structure. Note PHOT1::GFP fluorescence from the two pairs of guard cells where they meet (arrows) (Bar = 20 μm).

(C) Computer-reconstructed cross-sectional image of guard cells. This image was produced with ImageJ software by vertically slicing the original z-series dataset—a consecutive series of longitudinal optical sections. Cross-section of epidermis of 4 d old seedling showing guard cells (top center of image). PHOT1::GFP fluorescence is strongest from the anticlinal walls of the guard cells. Note that the adjacent epidermal cell to the right lacks fluorescence from its inner periclinal wall (Bars = 20 μm).

(D) Computer-reconstructed cross-sectional image of cotyledon epidermis. Image produced as in Figure 3C. Cross-section of epidermis and first layer of cortical cells. PHOT1::GFP fluorescence is detectable only on the anticlinal walls of the epidermal cells, the anticlinal walls of the underlying cortical cells, and the common walls between them. At this resolution, it is not possible to determine whether the fluorescence at the common wall is from PHOT1::GFP at the outer periclinal wall of the cortical cells, the inner periclinal wall of the epidermal cells, or both.

(E) Projection image of abaxial face of cotyledon of 3 d old seedling. Image was constructed from a z-series of images from the surface of the cotyledon to a depth of 30 μm . Note lack of PHOT1::GFP expression at the inner walls in the guard cells (arrow). Cf. Figure 3B (Bars = 20 μm).

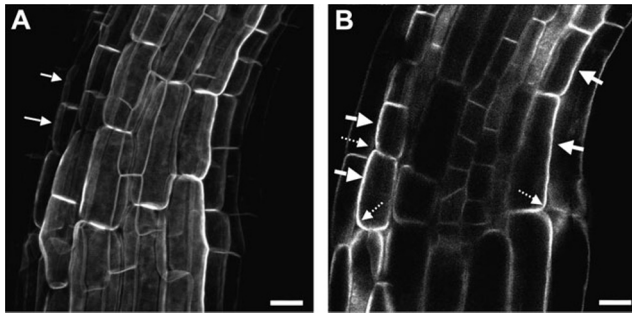


Figure 4. Localization of PHOT1::GFP in the Apical Hook Region. **(A)** Brightest point projection of optical sections from the surface of the hook to a depth of 50 μm . Note smooth labeling of surfaces of underlying cortical cells, polar distribution of signal in both epidermal and cortical cells, with strong fluorescence arising from the region of contact between cortical and epidermal cells. Outermost epidermal walls weakly labeled (thin arrows). **(B)** Single image chosen from z-series images. Note 'C'-like images of cortical cells (thick arrows). Prevascular tissue is shown weakly in the center of the image, and also shows somewhat polar distribution of PHOT1::GFP fluorescence. Where there is clear physical separation of cortical cells from epidermal cells, it is obvious that the strong signal at the common faces arises from the cortical cells and not the epidermal cells (Figure 4B, dashed arrows) (Bar = 20 μm).

signal (Figure 5B). (The exceptionally bright labeling of cell borders that are vertical to the optical axis of scanning are likely an optical artifact.) The pattern of labeling in the shoot at the transition zone is similar to the pattern seen in the elongation zone (Figure 5D).

Distribution in Elongating and Mature Hypocotyl Tissues

In both the hypocotyl elongation and maturation zones, as in the hook, the epidermal signal is considerably weaker than that in the cortical cells (Figure 6A and 6B). The pattern of heavy signal seen at the outer periclinal wall and the anticlinal walls of the cortical cells adjacent to the epidermis of the hook, forming a 'C' in longitudinal section, is not as distinct in the elongation zone, although the 'C' shape is still evident (Figure 6A, arrows). In the elongating and mature regions, the cortical cells have moderate signal on all faces (Figure 6B). There remains a tendency toward bipolar distribution of the signal, although the polarity appears not as strong in our images of cortical cells as previously reported by Sakamoto and Briggs (2002). As in the hook, some GFP fluorescence is present in the inner tissues (Figure 6B). These inner cells, possibly vascular parenchyma, show a tendency toward polar distribution of signal (thin arrows).

Distribution in the Shoot–Root Transition Zone

As noted above, there is strong expression of GFP fluorescence detectable in the shoot–root transition zone (see Figure 1A and 1B). Whether the strong expression reflects a real increase in PHOT1::GFP gene expression in this region or simply appears because these cells have not elongated as much as their coun-

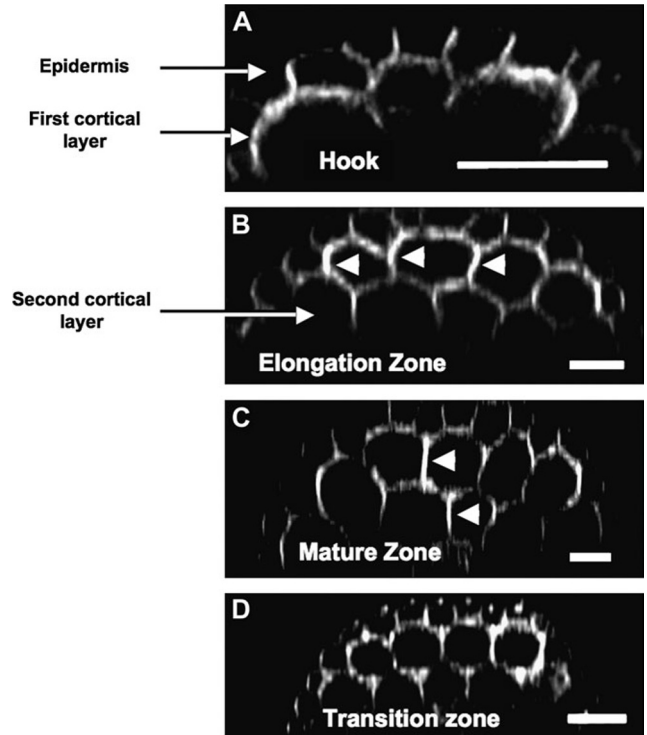


Figure 5. Computer-Reconstructed Cross-Sectional Images of Hypocotyl Cortex and Epidermis.

These images were produced with ImageJ software by vertically slicing the original z-series dataset—a consecutive series of longitudinal optical sections.

(A) Hook region. Note absence of PHOT1::GFP at inner periclinal walls of cortical cells, outer periclinal walls of epidermal cells, strong signal on outer periclinal and anticlinal walls of cortical cells. **(B)** Elongation region. Note continued absence of signal at outer periclinal walls of epidermal cells; fairly complete signal of first underlying layer of cortical cells with stronger signal on anticlinal than periclinal walls; and labeling of anticlinal walls of next cortical layer in. **(C)** Mature hypocotyl. The outer periclinal walls of epidermal cells show a trace of signal and both outer and inner cortical cells fairly completely labeled with strongest signal on anticlinal walls (thick arrows). **(D)** Shoot side of shoot/root transition region. All cell types including the epidermal cells appear fairly uniformly labeled on all sides (Bar = 50 μm).

terparts above them, or both, is an open question. Both cortical and epidermal cells on the shoot side show the same pattern of fluorescence as the hypocotyl cells above them— weaker in the epidermis than in the cortex, with only slight polarity in both cases (Figure 7A and 7B). The transition from shoot to root is marked by a dramatic and abrupt overall decrease in signal. Note that the weak GFP fluorescence in the shoot epidermal cells is completely absent from the root epidermal cells. The elongated root cortical cells show only very weak signal, largely concentrated at their end walls in a highly polar fashion (Figure 7, arrows).

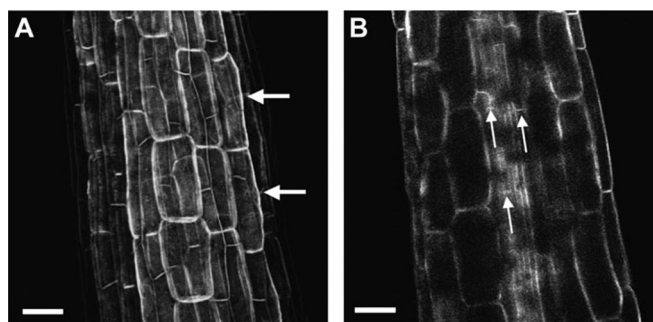


Figure 6. Localization of PHOT1::GFP in the Elongation Zone.

(A) Brightest point projection of optical sections from the surface of the hook to a depth of 80 μm . Epidermal cells (weaker signal) show uniform distribution on their anticlinal and outer periclinal walls; cortical cells (stronger signal) show more uniform labeling and far less polarity on all of their walls than in the hook region. Hence, the 'C' image is weaker than in the hook (arrows).

(B) Single image chosen from z-series. Epidermal cells are uniformly labeled and the inner periclinal walls of the outer cortical cells are now also labeled so that the 'C' image is weaker (arrows). The developing vascular strand (center) shows fairly heavy signal with a suggestion of stronger fluorescence from the end walls.

Distribution in Mature and Elongating Root Tissues

As shown by Sakamoto and Briggs (2002), the epidermal cells of roots of 4 d old etiolated seedlings lack any detectable expression of *PHOT1::GFP*. The absence of PHOT1::GFP in the root epidermis is even more dramatically illustrated in images of roots stained with the red-fluorescing membrane stain FM4-64 (Ovecka et al., 2005) (Figure 8A–8D). Not surprisingly, root hairs, of epidermal origin, also fail to express PHOT1::GFP (Figure 8B, arrows). The root cortical cells show polar distribution of PHOT1::GFP through the mature zone (Figure 7A) and into the elongation zone (Figure 8B). The central stele is also labeled as indicated by the diffuse fluorescence seen in all four images in Figure 8. Again, technical limitations (e.g. light scattering and self-shading) prevented resolving individual cell types. The elongating cortical cells closer to the root tip also show a strong bipolar distribution of signal (Figure 8B–8D), as previously described by Sakamoto and Briggs (2002).

Distribution in the Apical Millimeter of the Root

As shown in Figure 8C, there is relatively strong signal in the elongating region basal to the root cap. In the more mature region of the root tip, the most intense fluorescence arises from the stele (Figure 8B). The red fluorescence from the membrane stain FM4-64 clearly defines the epidermis in Figures 8B–8D, verifying a lack of detectable GFP signal from these cells. As shown in Figure 8D, signal is also undetectable within either the root cap or root apical meristem in these 4 d old seedlings. Signal first appears in cells destined to become cortex and endodermis, and only appears more basally in the inner prestelar tissues (Figure 8C and 8D). In parallel with the absence of signal at any face of the epidermal cells, there is only a trace of signal

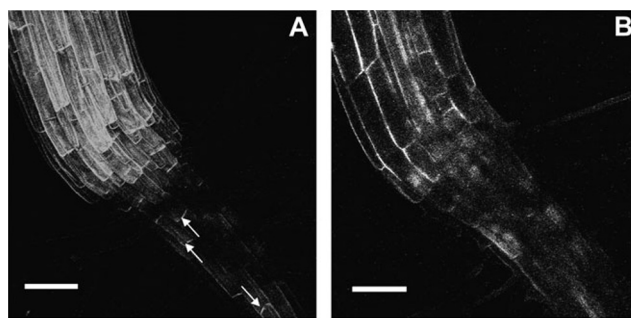


Figure 7. Localization of PHOT1::GFP in the Shoot–Root Transition Zone.

(A) Brightest point projection of optical sections from the surface of the hook to a depth of 100 μm . Cortical (strong fluorescence) and epidermal (weak fluorescence) cells on the shoot side are relatively uniformly labeled on all walls. Cortical cells on the root side, by contrast, show sharp polar distribution (arrows).

(B) Single median image from z-series showing somewhat polar PHOT1::GFP distribution in vascular strand in addition to that in cortical cells. Note complete absence of PHOT1::GFP expression in root epidermis (Bar = 100 μm).

at the outer periclinal walls of the cortical cells. However, their inner periclinal walls adjacent to incipient endodermal cells share strong signal with the adjacent endodermal cells and their anticlinal walls are also strongly labeled, giving them once again the appearance of a 'C', but this time with the open side facing out (Figure 8C and 8D). The smaller endodermal cells also show a 'C'-shaped pattern, with shared heavy signal in common with the cortical cells and at their anticlinal walls. There is only weak signal at their inner periclinal walls adjacent to the prestelar tissue. Hence, the opening of their 'C' faces inward. At this time, it has not been possible to determine whether the signal on the common walls between cortex and endodermis is endodermal, cortical, or both.

Blue Light-Induced Re-localization of PHOT1::GFP

As shown previously (Sakamoto and Briggs, 2002), blue light induces a re-localization of PHOT1::GFP into the cytoplasm. They observed the phenomenon in both cortical and epidermal cells in the hypocotyl hook region. Here, we have investigated the phenomenon at high resolution in a range of cell types in cotyledons, hypocotyls, and roots. We have also investigated the minimum requirement to induce re-localization and the onset of re-localization as a function of total blue-light fluence. Preliminary experiments showed that neither red nor green light induced any re-localization.

Hypocotyl Cells

The blue-light-induced changes in PHOT1::GFP localization appears as a dramatic reorganization at the cell cortex and detection of signal in the cytoplasm clearly internal to the cell volume. Figure 9A–9C shows changes occurring at the cell

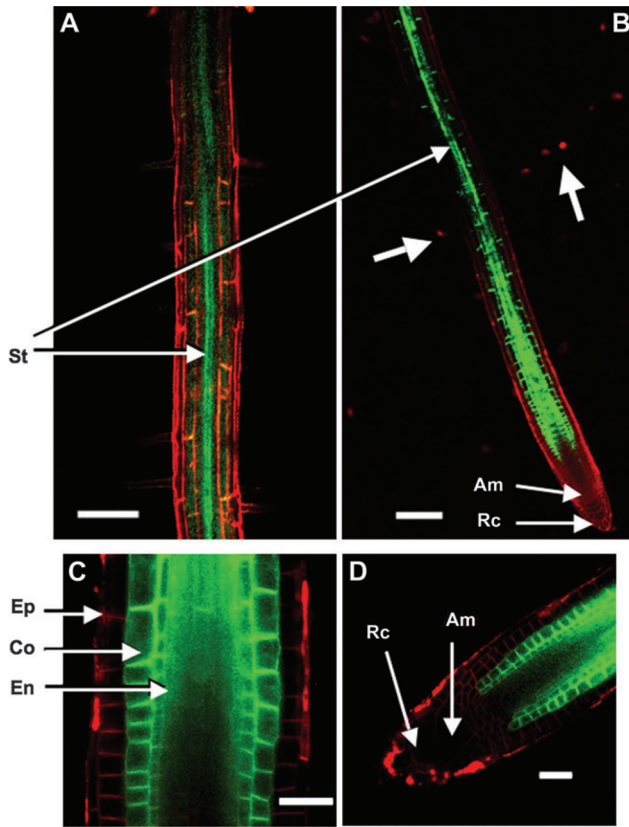


Figure 8. Distribution of PHOT1::GFP in Roots of 4 d Old Etiolated Seedlings.

(A) Single low-magnification confocal section of mature root-hair zone. Mature root-hair zone. Note heavy expression of PHOT1::GFP along stele and polar distribution of signal in cortical cells, indicated by co-localization of PHOT1::GFP (green) and FM4-64 (red) to give a yellow color. Root hairs labeled only with FM4-64 are faintly visible (thick arrows). Am, apical meristem; Co, cortex; En, endodermis; Rc, root cap; St, stele (Bar = 100 μm).

(B) Single low-magnification confocal section of root-tip zone. Note heavy PHOT1::GFP expression in prevascular tissue and cortex, persisting in vascular region and showing distinct polar distribution in cortical cells. Epidermis shows signal only from FM4-64 and not from PHOT1::GFP (Bar = 100 μm).

(C) Single high-magnification confocal section of root-tip elongation zone. Note lack of PHOT1::GFP signal in epidermal cells (FM4-64 red fluorescence only). Cortical cells heavily labeled at periclinal walls, only lightly labeled on the side toward the epidermis. Endodermal cells heavily labeled on anticlinal walls, lightly labeled on inner periclinal walls adjacent to stele. Whether heavy signal between endodermal and cortical cells arises from cortical or endodermal cells or both could not be resolved (Bar = 20 μm).

(D) Single high-magnification confocal section of extreme root tip. Labeling of cortex and endodermis precedes labeling of prestelar tissue. Note complete absence of detectable PHOT1::GFP fluorescence from root cap and apical meristem. First detectable fluorescence appears in elongating cortical and endodermal cells (Bar = 20 μm).

cortex of several cortical cells in the hypocotyl elongation zone during their exposure to the blue light from the scanning laser ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$). At time zero, PHOT1::GFP was smoothly distributed over the entire cell surface. The line down the middle represents the wall of an unlabeled epidermal cell overlying the cortical cell (Figure 9A). After 10 min of continuous blue-light treatment from the confocal microscope scanning laser, the uniformity once seen at the cell surface was drastically altered and the signal took on a mottled distribution with distinct dark areas outlined by more intense signal (Figure 9B, arrows) and punctuate structures became evident. After 60 min, the mottling increased and more punctuate structures and dark circles were clearly visible (Figure 9C). Two movies showing these changes are available on the web.

These dramatic changes were accompanied by detection of PHOT1::GFP in the cytoplasm. Figure 9D–9F shows a single section through the outermost cell layers (epidermis and cortex) from the elongating region of the hypocotyl. Again, the blue-light source is the confocal microscope laser. Already, after 5 min of scanning with blue light, what appear to be strands and blebs of PHOT1::GFP fluorescence were visible in the cytoplasm in both epidermal and outer and inner cortical cells (Figure 9E, arrows). At 10 min, further changes in the intracellular distribution of signal in the two cell types are visible (Figure 9F).

Induction of these distributional changes of PHOT1::GFP is very sensitive to blue light. The transgenic seedlings were exposed to three different intensities of blue light for 10 min prior to observation in the confocal microscope immediately thereafter. A total fluence of $300 \mu\text{mol m}^{-2}$ was insufficient to induce any detectable change within that time (Figure 10A). However, $600 \mu\text{mol m}^{-2}$ was sufficient to induce major changes in PHOT1::GFP distribution (Figure 10B, arrows) and $3,000 \mu\text{mol m}^{-2}$ likewise induced dramatic changes (Figure 10C, arrows). Figure 9A provides a suitable dark control.

When seedlings were exposed blue light for 30 min ($10 \mu\text{mol m}^{-2} \text{s}^{-1}$) and then left in darkness for 1 h, there was still major reorganization of PHOT1::GFP in cortical cells in the hook region (Figure 11A). Empty areas and small punctate structures are clearly evident in this image (arrows). However, after 2 h of darkness (Figure 11B), the signal assumed a more uniform distribution in several cells (thin arrows), although a few punctate structures and empty areas (thick arrows) persisted.

The time of onset of the re-localization process is a function of the total fluence of the initial blue-light stimulus (Figure 12). Dark-grown seedlings were given total fluences ranging from 100 to $10,000 \mu\text{mol m}^{-2}$, all given over a period of 100 s. A new seedling from each treatment was examined every 5 min in the confocal microscope to detect the first evidence for re-localization. When the total fluence was only $100 \mu\text{mol m}^{-2}$, no significant response had occurred after 100 min (Figure 12A). At 10 times the fluence, re-localization was first detectable after 45 min (Figure 12B, arrow); at 20 times the fluence, after 20 min (Figure 12C, arrow); and after 100 times the fluence, after 5 min (Figure 12D, arrow). Hence,

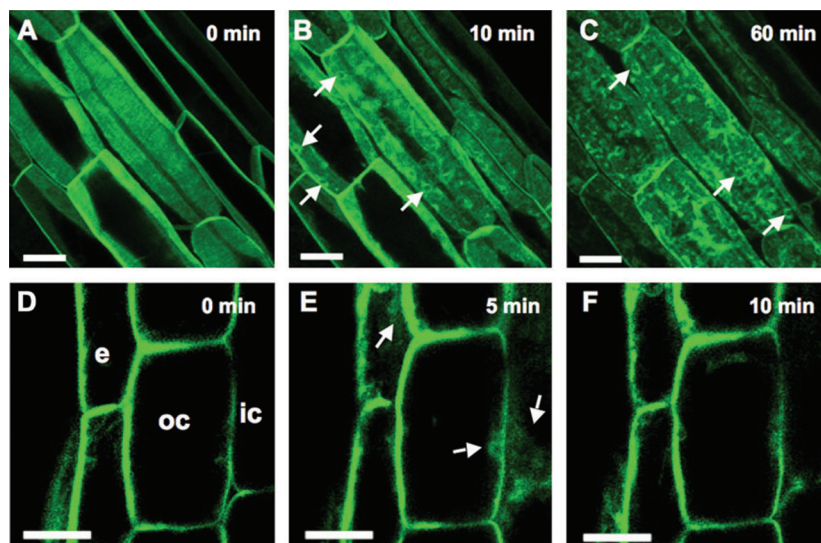


Figure 9. Blue-Light-Induced Changes in PHOT1::GFP Distribution in Cortical Cells.

Changes in PHOT1::GFP distribution in cortical cells from the elongation region of the hypocotyl with time of blue-light treatment. (A–C) Brightest point projection showing changes on the plasma membrane at the outer periclinal cell wall of a cortical cell during blue-light treatment. Line down center of Figure 9A likely is area out of the field of the image. Arrows indicate circular areas devoid of signal. (D–F) Single images through cortical cells in same region showing movement of PHOT1::GFP fluorescence into the cytoplasm. e, epidermis; oc, outer cortex; ic, inner cortex. Arrows indicate PHOT1::GFP in cytoplasm (Bar = 20 μm). Blue-light source, scanning laser (25 $\mu\text{mol m}^{-2} \text{s}^{-1}$).

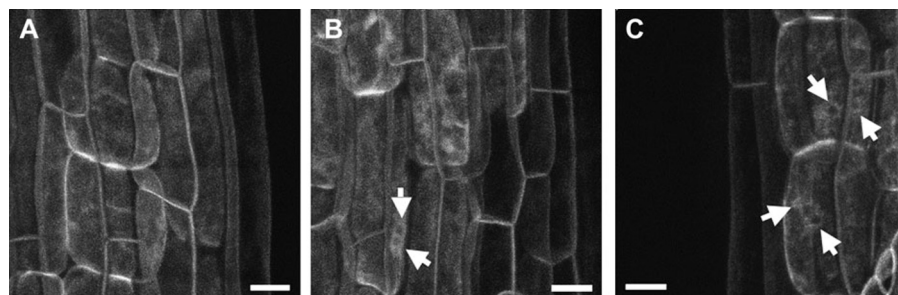


Figure 10. Sensitivity of PHOT1::GFP Re-localization in Hypocotyl Cortical Cells to Blue Light.

Brightest point projections. Etiolated seedlings were exposed to blue light for 10 min at 0.5 (A), 1 (B), or 5 (C) $\mu\text{mol m}^{-2} \text{s}^{-1}$ (total fluences 300, 600, and 3000 $\mu\text{mol m}^{-2}$, respectively). Note circular areas devoid of signal in B and C (arrows). Blue-light source, halogen lamp plus blue filter (Bar = 20 μm).

the greater the initial fluence is, the earlier the onset of the reorganization. The time of onset decreases approximately with the log of fluence, suggesting that the response is the consequence of a first-order photoreaction.

Cotyledon Cells

Blue-light-activated PHOT1::GFP re-localization is also found in both the epidermal and mesophyll cells of the cotyledon. Figure 13 shows images of the surface near the cotyledon margin in single optical section (A–C). As early as 6 min after the beginning of scanning (25 $\mu\text{mol m}^{-2} \text{s}^{-1}$), changes are visible in both cell types. The surfaces of the underlying mesophyll cells show the beginning of some mottling and there are small

particulate structures within the epidermal cells (Figure 13B). After 12 min of scanning, there is further release of PHOT1::GFP signal into the cytoplasm of the epidermal cells (Figure 13C) and the mottling on the upper surface of the mesophyll cells has increased.

Examination of guard cells on the cotyledons of 4 d old etiolated seedlings shows that they are unique among cell types examined in that they do not undergo a similar re-localization of PHOT1::GFP following blue-light treatment (not shown). It may be that these guard cells are not fully functional at this stage of development. Alternatively, light-activated release of PHOT1::GFP into the cytoplasm simply may not occur in this cell type.

Root Cortical Cells

Because the PHOT1::GFP signal is weaker in the root tissues than in those of the hypocotyl and cotyledon, it was not possible to see changes that might be occurring at the plasma membrane itself, comparable to those shown in Figure 9A–9C. However, movement of the signal into the cytoplasm following blue-light treatment is easily observed. Figure 14A–14C shows the changes in distribution of green fluorescence after 0, 6, and 12 min following excitation with the scanning laser. The signal moves through what appear to be cytoplasmic

strands that converge on a layer of cytoplasm surrounding the nuclear region (arrows, B, C). Neutral red staining indicates that this region is approximately the same size as the nucleus and in the same position (data not shown). Under higher power (Figure 14D), some small punctuate structures can be observed (arrows).

The sensitivity of root cells to blue light appears similar to that found in hypocotyl cortical cells (Figure 14E–14G). Without blue-light treatment, no cytoplasmic PHOT1::GFP is detectable (Figure 14E). Ten minutes of exposure to a total fluence of 600 $\mu\text{mol m}^{-2}$ is sufficient to induce small changes (Figure 14F). However, 3000 $\mu\text{mol m}^{-2}$ induces clearly detectable migration of signal into the cytoplasm (Figure 14G, arrows).

As is the case with the hypocotyl cortical cells, cytoplasmic PHOT1::GFP in root cortical cells disappears in darkness following blue-light treatment. As shown in Figure 15D, the cytoplasm shows little signal after 1 h of darkness following a 30-min blue-light exposure (10 $\mu\text{mol m}^{-2} \text{s}^{-1}$). In root cortical cells, dark recovery appears somewhat more rapid than in hypocotyl cortical cells (cf. Figure 11).

Blue Light-Induced Movement and Dark Recovery in the Presence of a Protein Synthesis Inhibitor

Roots of 4 d old etiolated seedlings were treated with 50 μM cycloheximide for 30 min in darkness prior to observation in the confocal microscope (Figure 15A). This concentration, administered to 3 d old etiolated seedlings for 30 min, completely prevented the immediately subsequent incorporation of ^{35}S -methionine into protein (results not shown). Migration of PHOT1::GFP into the cytoplasm in root cortical cells in the elongation region is clearly evident after 10 min of scanning (blue light 25 $\mu\text{mol m}^{-2} \text{s}^{-1}$) (Figure 15B, arrows) and even

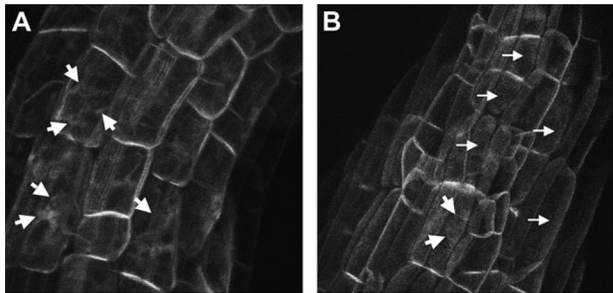


Figure 11. Disappearance of PHOT1::GFP from Cytoplasm of Hypocotyl Cortical Cells with Time in the Dark.

Brightest point projections. Etiolated seedlings were exposed to blue light (10 $\mu\text{mol m}^{-2} \text{s}^{-1}$) for 30 min before being returned to darkness.

(A) Appearance after 1 h darkness. Some mottling and dark circular areas (arrows) still visible.

(B) Appearance after 2 h. Signal at many cell surfaces mostly smooth (thin arrows), although a few empty circular areas persist (thick arrows). Blue-light source, halogen lamp plus blue filter (Bar = 20 μm).

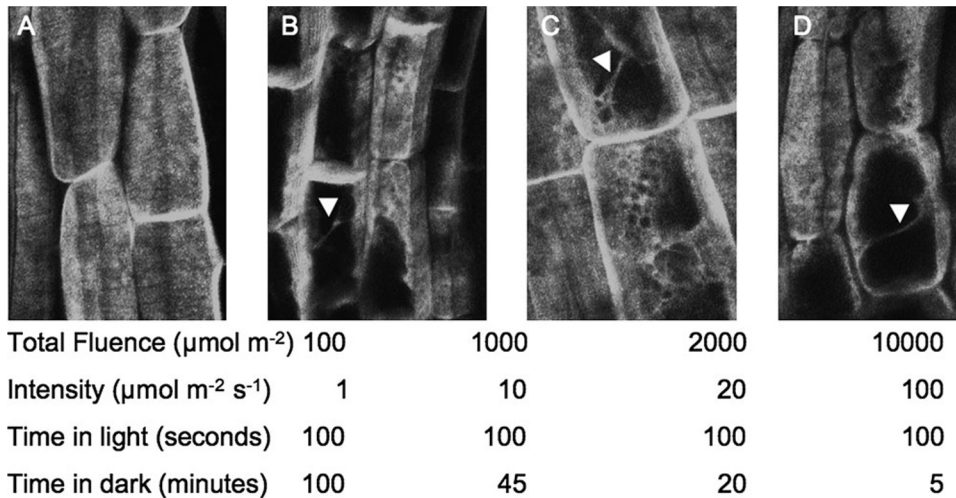


Figure 12. Blue-Light-Induced Re-localization of PHOT1::GFP in Hypocotyl Cortical Cells is Sensitive to Fluence Total fluences: (A) 100, (B) 1,000, (C) 2,000, (D) 10,000 $\mu\text{mol m}^{-2}$.

Time in dark is time in darkness between blue-light pulse and observation in the confocal microscope. Brightest point projections. Etiolated seedlings were exposed to blue light of various intensities for 100 s then returned to darkness. At 5-min intervals, hypocotyl cortical cells were examined in the confocal microscope for evidence of PHOT1::GFP re-localization (arrows).

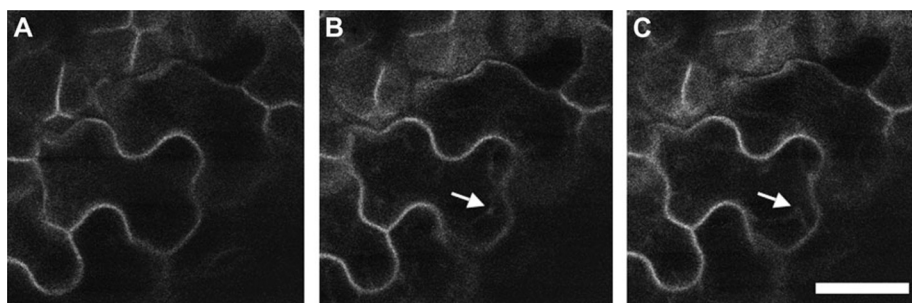


Figure 13. Blue-Light-Induced Re-localization of PHOT1::GFP in Cotyledon Epidermal and Mesophyll Cells.

(A–C) Single scanning images through epidermal cells. (A) zero time; (B) 6 min of scanning; (C) 12 min of scanning. The three images show appearance of signal in the cytoplasm of epidermal cells with time (arrows, B, C). Blue light source, confocal microscope laser (Bar = 20 μm).

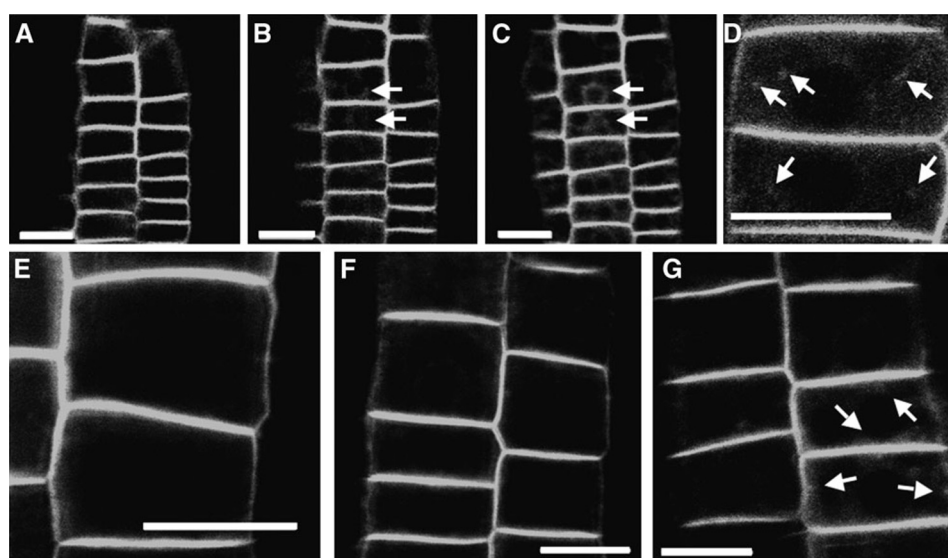


Figure 14. Blue-Light-Induced PHOT1::GFP Re-localization in Root Cortical Cells.

Single confocal sections of root cortical cells.

(A–C) Zero, 6, and 12 min of blue-light exposure, respectively. Blue-light source, scanning laser ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$).

(D) High-power image showing punctuate structures in cytoplasm. Note heavily labeled end walls (A, D) and signal surrounding the nuclear region (B, C). Blue-light source, scanning laser ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$).

(E–G) Sensitivity of root cortical cells to blue light. Etiolated seedlings were exposed to blue light for 10 min at 0.5 (E), 1 (F), or 5 (G) $\mu\text{mol m}^{-2} \text{s}^{-1}$ (total fluences 300, 600, and 3000 $\mu\text{mol m}^{-2}$, respectively). Arrows in G indicate cytoplasmic PHOT1::GFP. Blue-light source, halogen lamp plus blue filter (Bar = 20 μm).

more dramatic after 30 min (Figure 15C, arrows). Hence, the re-localization itself brought on by blue light does not require protein synthesis.

When the roots were kept in darkness following 30 min of blue-light treatment ($10 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 1 h (Figure 15D), in the absence of cycloheximide, PHOT1::GFP gradually decreased from the cytoplasm. In the presence of cycloheximide, the signal also disappeared (Figure 15E, 1 h darkness; Figure 15F, 2 h darkness). Hence, the disappearance from the cytoplasm may not require protein synthesis. Whether the disappearance results from return of the cytoplasmic PHOT1::GFP

to the membrane or its degradation cannot be resolved at this time. However, the disappearance from the cytoplasm was considerably slower than in the absence of the inhibitor (cf. Figure 14D and 14E). Given the length of the incubation in cycloheximide, perhaps this result is not surprising.

Relative Phototropic Sensitivity of Wild-Type and *phot1GFP* Seedlings

Before we could attempt to relate blue-light-induced changes in PHOT1::GFP to any physiological responses in wild-type plants, it was necessary to determine the relative physiological

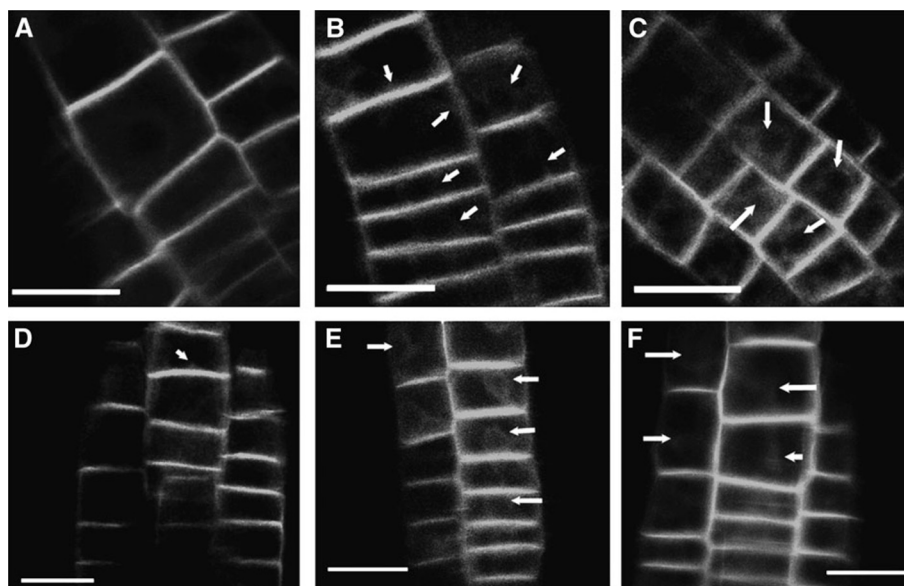


Figure 15. Effect of Cycloheximide on Blue-Light-Induced PHOT1::GFP Re-localization and Subsequent Recovery in Darkness.

(A) Root cortical cells after 30 min cycloheximide treatment in darkness.

(B) Root cortical cells after 30 min cycloheximide treatment in darkness and 10 min blue-light treatment at 10 μmol m⁻² s⁻¹. Cytoplasmic PHOT1::GFP is faintly visible.

(C) Root cortical cells after 30 min cycloheximide treatment and 30 min blue-light treatment at 10 μmol m⁻² s⁻¹. Cytoplasmic PHOT1::GFP clearly visible, especially surrounding the nuclear region (arrows, B, C).

(D–F) Root cortical cells following 30 min of blue light (10 μmol m⁻² s⁻¹) and subsequent incubation in darkness in the absence of cycloheximide (D: 60 min) or in the presence of cycloheximide (E: 60 min, F: 120 min). Traces of signal after 2 h (F; cf. D) in the presence of the inhibitor indicate that the disappearance of signal in root cortical cells is considerably slower in the presence of cycloheximide than in its absence. Light source, halogen lamp plus blue filter (Bar = 20 μm).

sensitivity of the transgenic seedlings to blue light compared to wild-type seedlings. Sakamoto and Briggs (2002) showed that the levels of PHOT1::GFP protein in the transgenic seedlings were present at a level roughly equivalent to those for *phot1* itself in wild-type seedlings. They also showed that the *PHOT1::GFP* transgene restored a strong phototropic response in the null mutant *phot1-5* (*nph1-5*) to a fluence rate of 2 μmol m⁻² s⁻¹ for 24 h. The fluence rate was chosen to be insufficient to activate *phot2* (Sakai et al., 2001). However, these conditions are clearly saturating (70° curvature for the transformant and a little over 80° for wild-type) and therefore do not give any information on sensitivity with respect to wild-type seedlings.

We therefore exposed both wild-type seedlings and seedlings expressing the *PHOT1::GFP* gene driven by the *PHOT1* promoter to 2 μmol m⁻² s⁻¹ unilateral blue light for different periods of time to obtain a time course for the development of curvature in each case. The results are shown in Table I. Evidently the level of expression of the transgene is either insufficient for full complementation of phototropism for the *phot1-5* mutation or the labeled protein is not fully functional. Expression of GFP-tagged *phot1* protein is nevertheless sufficient for at least partial PHOT1 function and to allow confocal

Table 1. Complementation of Phototropism in Etiolated *Arabidopsis* Hypocotyls by PHOT1::GFP.

Exposure time (h)	PHOT1::GFP	Wild-type
6	19.0 ± 1.5	40.8 ± 3.7
24	49.7 ± 4.5	74.1 ± 4.1

Blue-light fluence rate 2 μmol m⁻² s⁻². *n* > 50. S.E.M., standard error of the mean. Degrees curvature with S.E.M.

examination of light-induced changes in PHOT1::GFP distribution and re-localization.

DISCUSSION

PHOT1::GFP Tissue Distribution Patterns with Respect to Physiological Responses

The distribution of PHOT1::GFP in different tissues is largely consistent with its physiological role. Thus, it is not surprising to find it in guard cells of the cotyledon where *phot1* mediates stomatal opening (Kinoshita et al., 2001). Likewise, given that the epidermis is likely the limiting factor in leaf expansion (Van Volkenburgh, 1999), it is not surprising to find it in epidermal

cells. Given its role in phototropism, the strong expression found in the hypocotyl hook and elongation zone is to be expected, as is the strong expression in the root elongation zone. Its distribution in mesophyll cells is consistent with its role in mediating the chloroplast avoidance (Jarillo et al., 2001; Kagawa et al., 2001) and accumulation (Sakai et al., 2001) responses. Sakamoto and Briggs (2002) had already noted a lack of detectable expression either in the root cap or the root apical meristem, although they reported a faint signal from the epidermis. In the present study, we were unable to detect PHOT1::GFP expression in the root epidermis. A difference in seedling age or growth conditions might account for this discrepancy.

The literature on the site of root photosensitivity for phototropism indicates that the response is complex. Mullen et al. (2002) presented evidence that the root cap of maize seedlings is the site of photosensitivity for root phototropism. White light applied via optic fiber to the root tip induced curvature, whereas white light applied similarly to the elongation zone failed to produce a response. Liscum and Briggs (1995) demonstrated that it was phot1 that mediated phototropism in response to low-fluence blue light in *Arabidopsis* roots. Since PHOT1::GFP appears absent from the root cap and apical meristem in 4 d old etiolated seedlings, either *Arabidopsis* and maize use different systems to regulate root phototropism or photoexcitation of some other photoreceptor in the root cap of *Arabidopsis* is required for phototropin distal to the root cap to become functional. Light piping, known to occur in etiolated tissues (Mandoli and Briggs, 1982) and light scattering, could result in a light gradient in the elongation zone, even if only the root cap were directly illuminated but could not account for the greatest photosensitivity occurring in the cap region. We recently tested PHOT1::GFP seedlings for their negative phototropic response, inspecting them before and after 8 h phototropic induction with $2 \mu\text{mol m}^{-2} \text{s}^{-1}$ of blue light. The roots clearly curved away from the light in the absence of detectable PHOT1::GFP fluorescence in their apical meristems or root caps before or after phototropic stimulation (Inseob Han, Margaret Olney, and Winslow R. Briggs, unpublished).

As Mullen et al. (2002) suggest, perhaps the activation of phytochrome, known to be located almost exclusively in the root-cap columella cells in roots of maize and other etiolated grass seedlings (Pratt and Coleman, 1974), is required to potentiate a tropic response to blue light in the phototropin-rich elongation region. Blue light can transform phytochrome *in vivo*, although the Pfr:Pr ratio is small and the quantum efficiency low compared to these parameters in red light (Pratt and Briggs, 1966). As Mullen et al. (2002) used continuous blue light, phytochrome activation certainly must have occurred.

It is not clear why PHOT1::GFP expression should be high in the root-shoot transition region. Galen et al. (2007) have reported that phot1 serves a role in drought tolerance in field-grown *Arabidopsis*. Even if the zone is under the soil, light piping could provide sufficient phototropin activation to induce some sort of response (Mandoli and Briggs, 1982). Under

drought stress, blue-light-activated phot1 in the transition region might send signals to the roots that could modulate their growth rate or growth direction. Clearly, further investigation is needed to clarify the role (if any) of phot1 in this region.

PHOT1::GFP Subcellular Distribution

With the higher-resolution confocal microscopy now available to us, we were able to discern a great deal more subcellular detail than that found in the previous study by Sakamoto and Briggs (2002). The images presented above reveal features of PHOT1::GFP subcellular distribution patterns that we previously could not resolve.

Sakamoto and Briggs (2002) reported that blue-light-induced movement of PHOT1::GFP into the soluble fraction of the cytoplasm and Knieb et al. (2004) reported a similar result with the native phot1 in mustard seedlings. In both cases, the observed activity could have come from extremely small vesicles as well as from protein in solution. High-speed centrifugation was used to separate membrane and soluble proteins in both studies. Knieb et al. (2004) estimated that at least 20% of the total phot1 was released to the cytoplasm following blue-light treatment of etiolated mustard seedlings. Immunoblotting provided no evidence for any breakdown products in either study. Whether some of the cytoplasmic PHOT1::GFP that we observed is within endosomal vesicles is not resolved in the present study. These results are in contrast to those of Kong et al. (2006) with phot2. Blue-light-induced phot2 migration into vesicles that co-localized with a Golgi marker but the authors could not detect any soluble fraction after high-speed centrifugation.

It has been well established for a number of plant species that phot1 is closely associated with the plasma membrane in dark-grown seedlings (see Briggs et al., 2001 for references). Results from the present study are consistent with that view. In dark-grown seedlings, PHOT1::GFP fluorescence is found closely associated with the outermost surface of the cytoplasm in all cell types in which we found it expressed. What is surprising is that different cell types and similar cell types at different stages of development may show unique patterns. In the cotyledons, distribution appears uniform at the plasma membrane of mesophyll cells but is limited almost entirely to the anticlinal walls of the epidermal cells (Figure 3). Little, if any, signal was detected on the periclinal walls. Expression in the marginal epidermal cells was especially weak compared to the cells on the abaxial surface of the cotyledons (Figure 3A). Like other epidermal cells, the guard cells also have PHOT1::GFP largely on their anticlinal walls. However, it appears later in guard cells than in the other epidermal cells (cf. Figure 3B and 3E) and shows particularly strong expression on the contacting walls between the two guard cells of a single stomate (Figure 3B). In the dividing tissue at the cotyledon margins, there is also exceptionally strong expression at what appear to be recently laid down cell walls of the mesophyll cells (Figure 3A). It is unclear how this expression might be related to cotyledon growth and physiology.

In the hook and elongation zone, there is strong expression at the anticlinal and outer periclinal walls of cortical cells, moderate expression on the end walls of elongating epidermal cells, and weak expression at their outer walls (Figures 4 and 5). The 'C'-shaped pattern seen for the outer cortical cells as visualized in longitudinal section is less apparent in elongating and mature tissues as the cell surfaces become more completely labeled, and the 'C'-shaped pattern shifts to the next cortical cell layer in.

Blue-Light Effects on PHOT1::GFP Subcellular Distribution

The function of blue-light-induced re-localization of phot1 and its movement into the cytoplasm is not clear. It could be a mechanism to desensitize plant tissues to blue light by removing photoreceptor from the region of the plasma membrane, where it is required to interact with other plasma-membrane-associated proteins. As mentioned in the Introduction, blue light induces some major ionic changes in plant tissues. Fuchs et al. (2003) reported the unilateral blue-light-induced gradient of expression for *ZMK1*, encoding a potassium channel, across a unilaterally illuminated maize coleoptile. However, the gradient developed rather slowly, first detected after 60 min irradiation and unlikely to be related directly to blue-light-induced changes in subcellular distribution. Cho and Spalding (1996) reported a rapid transient depolarization in *Arabidopsis* hypocotyls exposed to blue light attributable to activation of anion channels. This transient response occurred well before we observed the first changes in PHOT1::GFP distribution. Whether these changes bear a causal relation to subsequent changes in phot1 distribution remains to be determined.

There are ionic changes with kinetics similar to those of the blue-light-mediated PHOT1::GFP re-localization and recovery described here. Baum et al. (1999) reported rapid but transient increases in calcium uptake in response to a blue-light pulse by light-grown *Arabidopsis* seedlings expressing the calcium-sensing aequorin system. The overall changes lasted less than 100 s. However, a single blue-light pulse desensitized the system to subsequent pulses and recovery took place over 3–4 h, not unlike the dark recovery to a strict localization of PHOT1::GFP at the plasma membrane reported in the present study. Two groups have reported blue-light-induced increases in calcium uptake by etiolated tissues of *Arabidopsis* (Stoelzle et al., 2003) and several other dicot species (Babourina et al., 2003), respectively. Stoelzle et al. (2003) found the response strongly reduced in a *phot1-5* mutant and completely absent in a *phot1-5 phot2-1* double mutant. In both studies, the kinetics were consistent with those for blue-light-induced PHOT1::GFP re-localization, with the changes observable several minutes after the onset of blue light and continuing for 15 or more minutes thereafter. It is premature to do more than point out the correlation of these ionic changes with changes in the subcellular distribution of PHOT1::GFP. Further experimentation should determine whether there is a causal relationship between these changes in calcium movement and the loss of phot1

from the plasma membrane in blue light and its subsequent dark recovery to a plasma-membrane-only distribution.

PHOT1::GFP Distribution and Proteins Involved in Auxin Transport

Although phototropism is well documented to involve changes in auxin transport, there are several phototropin responses that do not. These include stomatal opening, solar tracking by leaves, and chloroplast movement. Thus, it is not surprising that the coincidence of PHOT1::GFP localization and that of the proteins involved in auxin transport—the PIN proteins (see Chen and Masson, 2006) and the ABC type transporters (multidrug-resistant P-glycoproteins or MDR/PGP auxin transport proteins; Blakeslee et al., 2006)—is not perfect. For example, PIN1::GFP in roots is located in the prevascular cylinder in roots and not in the endodermis, cortex, or epidermis, whereas PHOT1::GFP appears first near the root apex in cortex and endodermis and only later in the prevascular and vascular tissues. In addition, PIN1::GFP is located on the anticlinal walls distal to the shoot apex in the stem tissues and proximal to the root apex in roots. PHOT1::GFP in the stems is located at the cell walls both distal and proximal to the shoot apex and on the exterior periclinal wall as well. By contrast, PIN2::GFP in roots has a distribution that coincides closely with that of PHOT1::GFP. Both proteins appear in cortical and endodermal cells near the root apex (Xu et al., 2006). As is the case with PHOT1::GFP, PIN2::GFP shows a strong signal at the periclinal walls that form the contact between them, although it is not possible to discern whether the fluorescence arises from the cortical or endodermal cells, or both.

PIN3 was the first of the PIN proteins shown to have its distribution regulated by vesicular trafficking and to undergo a change in intracellular distribution under tropic stimulation—in this case, gravity (Friml et al., 2002). Subsequently, Blakeslee et al. (2004) demonstrated a redistribution of PIN1 in the hypocotyl hook under phototropic stimulation. The authors reported a light-induced gradient of PIN1 delocalization across the unilaterally illuminated hypocotyl (but not PIN3) after 1.5 h of irradiation with $0.5 \mu\text{mol m}^{-2} \text{s}^{-1}$. Curiously, the delocalization was strongest on the side of the hypocotyl away from the light, where the light fluence rate is presumably the lowest. However, the delocalization failed to take place in a *phot1*-null mutant, indicating that it required phot1. It will be important to determine how the rate of delocalization of PIN1 coincides with the re-localization of PHOT1::GFP in the same cells.

Currently, there is not general agreement on the exact roles played by the PIN proteins in auxin transport. Earlier reports referred to them as auxin efflux carriers but, with the discovery of the 21-member family of MDR/PGP proteins and their role in auxin transport, more recent papers refer to the PIN proteins as auxin transport facilitators (see Blakeslee et al., 2006). These proteins have somewhat specific but often overlapping distribution in different plant organs and tissues and show considerable redundancy. Whatever their precise function, their polar

localization clearly determines the direction of auxin transport in individual cells (Wiśniewska et al., 2006), and they have been shown to mediate auxin efflux from mammalian and yeast cell (Petrášek et al., 2006).

The MDR/PGP proteins also undergo membrane cycling and are known to stabilize specific PIN proteins (Blakeslee et al., 2006). At present, it is not known if their subcellular distribution in phototropically sensitive tissues is influenced by blue light. Clearly, studies with fluorescently labeled phot1, phot2, the various PIN proteins, and MDR/PGP proteins in various paired combinations will aid in elucidating the physical relationships between the auxin transport components and the two phototropins and the effects of blue light on these relationships.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

We used the *phot1-5* (*nph1-5*) mutant of *Arabidopsis thaliana* (cv. Columbia, *gl*-background) transformed with a *PHOT1::GFP* construct by Sakamoto and Briggs (2002). *PHOT1::GFP* expression was sufficient to complement the mutation to the extent that significant phototropic curvature was obtained after 24 h of unilateral blue-light treatment (Table I; Sakamoto and Briggs, 2002). The seeds were surface sterilized and planted on 0.4% phytoigel with half-strength Murishige and Skoog medium supplemented with 1% sucrose. The seedlings were grown in darkness for 4 d, except where otherwise noted, in a growth chamber at 22°C. All handling was done under dim green lights (Short et al., 1992), except as noted below.

Chemicals

The fluorescent red dye FM4-64 (Molecular Probes, Invitrogen) was dissolved in water (1 mg ml⁻¹) and stored at 4°C in a refrigerator. This stock solution was diluted to 5 μmol ml⁻¹ with half-strength Murishige and Skoog basal salts plus 1% sucrose for treatment of seedlings. Manipulations were carried out under the dim light from the computer screen. Seedlings were treated for 10 min in darkness prior to examination in the confocal microscope. Cycloheximide (Sigma) was used at a concentration of 50 μM. ³⁵S-methionine was obtained from Perkin Elmer. Seedling roots were treated to 50 μCi in 100 μL of half-strength Murishige and Skoog medium.

Confocal Microscopy

Figures 2–8 were obtained at the Institut für Physikalische und Theoretische Chemie at the University of Bonn, Germany, with a Zeiss LSM 510 Meta confocal microscope (Zeiss, Jena, Germany) using both a 20× air objective and a 63X, NA = 1.4 oil immersion lens. Figures 9–14 were obtained at the Carnegie Institution Department of Plant Biology, with a Biorad MRC1024 confocal microscope, using a Nikon 60× water-immersion objective, NA = 1.3. On both microscopes, excitation of GFP was performed at 488 nm. With the Biorad 30% transmittance neutral density filter in place, the fluence rate

at the sample with the 60×water-immersion objective lens was 25 μmol m⁻² s⁻¹. Fluorescence emission was measured between 505 and 530 nm for PHOT1::GFP fluorescence and at 650 nm for FM4-64 fluorescence. The external blue-light source used in some experiments was a halogen lamp (Phillips 20 MR 16, New Jersey, USA) passed through Corning glass filters: Corning number 5032 for blue light, number 4015 for green light, and number 2404 for red light.

Analysis of Whole Seedling Fluorescence

Fluorescence images of whole seedlings were obtained with a Leitz MZFLIII binocular microscope combined with a digital camera (Leica JVC KY-F708, Leitz, Wetzlar, Germany). Relative fluorescence intensities were measured with ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, Maryland, USA). Images were background subtracted and intensities of the different regions of the seedlings were measured for 10 seedlings and averaged for each region. The 'reslice' function of Image J allowed us to construct the cross-section images shown in Figures 3 and 5. The dimensions were calculated from the original z-series slices.

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