Assembly of Synthetic Locked Phycocyanobilin Derivatives with Phytochrome in Vitro and in Vivo in Ceratodon purpureus and Arabidopsis

Rui Yang, Kaori Nishiyama, Ayumi Kamiya, Yutaka Ukaji, Katsuhiko Inomata, and Tilman Lamparter

INTRODUCTION

Phytochromes are photoreceptors of plants, bacteria, and fungi that are most sensitive in the red and far-red spectral region (Schäfer and Nagy, 2006). In seed plants, many steps in the life cycle are under phytochrome control. The typical feature of phytochromes is their photoreversibility. In darkness, phytochrome is synthesized in the red light–absorbing Pr form, which is converted upon light absorption into the far-red-light–absorbing, physiologically active Pfr form. Light absorption of Pfr results in a photoconversion back to Pr. Hence, phytochrome responses are often maximally induced by red light, which favors Pr-to-Pfr photoconversion, and inactivated by far-red light, which favors the reverse photoconversion from Pfr to Pr (Smith, 1995). The model plant Arabidopsis thaliana, which is used in this study, contains five phytochromes, termed phyA to phyE. Prominent phytochrome-mediated responses are seed germination, hypocotyl elongation, cotyledon opening, and inhibition of gravitropism, which leads to a randomization of growth orientation (Kendrick and Kronenberg, 1994; Schäfer and Nagy, 2006). The other organism used in this study, the moss Ceratodon purpureus, contains four phytochromes, termed Phy1 to Phy4 (Thümmler et al., 1992; Hughes et al., 1996; Mittmann et al., 2009). Phy2 to Phy4 have a domain arrangement that is typical for plant phytochromes, whereas Phy1 is a unique fusion between a phytochrome N-terminal moiety and a C-terminal kinase. In Ceratodon filaments, phytochrome mediates phototropism and polarotropism, the gravitropism response, and the accumulation of chlorophyll and is involved in the formation of side branches (Lamparter, 2006), effects that can be studied at the level of a few individual cells.

In seed plants, three different types of phytochrome responses are distinguished: the very low fluence response (VLFR), the low fluence response (LFR), and the high irradiance response (HIR) (Casal et al., 1998). VLFRs are induced by very weak light, and the amount of Pfr produced by far-red pulses is sufficient to saturate such responses. HIRs require long irradiation times and are maximally induced by continuous far-red light. For Arabidopsis, it has been shown that Pfr is translocated from the cytosol to the nucleus (Nagatani, 2004), where the specific interaction with phytochrome interacting factor transcription factors can finally result in differential gene expression (Kikis et al., 2009). The VLFRs and HIRs are mediated by phyA, which accumulates during dark growth and becomes rapidly degraded in the light. However, phyA is absent in nonvascular plants and
fen (Schneider-Poetsch et al., 1998; Mathews, 2005; Mathews, 2010). Hence, the distinction between VLFR, LFR, and HIR is specific for seed plants and does not hold for, for example, mosses.

In recent years, studies on bacterial and fungal phytochromes have contributed to our current understanding of plant phytochrome action (Lamparter, 2004; Rockwell and Lagarias, 2010). Typical phytochromes have an N-terminal chromophore module, consisting of PAS GAF and PHY domains, and a C-terminal His-kinase or His-kinase-like module. The mechanism behind Pr/Prf photoreversibility seems universal, and biophysical studies on recombinant bacterial and plant phytochromes give detailed insight into protein structure and mechanisms behind phototropism activity. Based on structural studies of bacterial phytochromes, it is known that the bilin chromophore is embedded in a chromophore pocket that is predominantly formed by the GAF domain, and that Pas and GAF are interconnected in a knot structure (Wagner et al., 2005; Esser et al., 2008; Yang et al., 2008). The biliverdin (BV) chromophore of bacterial and fungal phytochromes is bound to a Cys residue in the PAS domain (Lamparter et al., 2002; Blumenstein et al., 2005), which is highly conserved in this group of phytochromes, whereas the phyco-cyanobilin (PCB) chromophore of cyanobacterial phytochromes and the phytochromobilin (PφB) chromophore of plant phytochromes are bound to a conserved Cys residue in the GAF domain (Rüdiger and Thümmler, 1994; Hübschmann et al., 2001). Amino acids in the region play important roles in plant phytochrome signaling and interaction with transcription factors (Kikis et al., 2009). It is generally agreed that the first step of the Pr-to-Pfr photocycle is a Z to E isomerization around the ring C–D–connecting double bond of the bilin chromophore (Rockwell and Lagarias, 2010; Song et al., 2011; Yang et al., 2011), although it has recently been proposed that a phytochrome-like protein from a thermophilic cyanobacterium undergoes motions around the ring A–B–connecting methine bridge (Uliljas et al., 2010).

Studies on the phytochrome chromophores as the central photoreceptor component contribute to a major extent to our present understanding of phytochrome action. By using inhibitors that block tetrapyrrole biosynthesis, it has been found that the phytochrome apoprotein incorporates the chromophore autocatalytically (Elich and Lagarias, 1987). Several mutants have been isolated in which chromophore biosynthesis is blocked. Characterizations of the Arabidopsis long hypocotyl mutants hy1 and hy2 (Parks and Quail, 1991) have led to the isolation of two key enzymes of PφB synthesis. HY1 encodes a heme oxygenase, which catalyzes the conversion of heme to BV (Muramoto et al., 1999), whereas HY2 encodes a phytochromobilin:ferredoxin oxidoreductase (referred to here as PφB synthase), which catalyzes the conversion of BV into the chromophore of plant phytochromes, PφB (Kohchi et al., 2001). Heme oxygenase mutants have also been isolated from several other plants, including the moss C. purpureus. Ceratodon class 1 phototropism (ptr) mutants are characterized by their aphototropic and low chlorophyll phenotype; both defects are rescued by BV (Lamparter et al., 1996; Brücker et al., 2005).

Phytochrome chromophore–deficient mutants of Arabidopsis and Ceratodon have been used for chromophore feeding studies. Besides BV, PCB, and PφB, the chromophore phycocerythrobilin (PEB) has been used, which is incorporated into apophytochrome and yields highly fluorescent adducts (Murphy and Lagarias, 1997; Böse et al., 2004), and the action of several synthetic chromophores has been tested in Arabidopsis (Hanzawa et al., 2001; Hanzawa et al., 2002). Recently, several synthetic locked chromophores have been assembled with bacterial phytochromes (Agp1 and Agp2) and Hanzawa et al. (2001, 2002). In this study, we used BV, PCB, and the locked 15ZaPCB and 15EaPCB (Figure 1) as chromophores. According to previous studies with locked BV derivatives, we expected that assembly with 15ZaPCB and 15EaPCB would yield photoinactive Pr- and Pfr-like adducts, respectively. We used three different recombinant phytochrome fragments as test systems for chromophore

RESULTS

In Vitro Studies: Assembly of Bacterial and Plant Phytochromes with BV, PCB, and Locked PCB Chromophores

In this study, we used BV, PCB, and the locked 15ZaPCB and 15EaPCB (Figure 1) as chromophores. According to previous studies with locked BV derivatives, we expected that assembly with 15ZaPCB and 15EaPCB would yield photoinactive Pr- and Pfr-like adducts, respectively. We used three different recombinant phytochrome fragments as test systems for chromophore
assembly: Agp1-M15 (the N-terminal 504 amino acids of Agrobacterium tumefaciens phytochrome Agp1, lacking the His-kinase) with the natural BV binding Cys at position 20; Agp1-M15CV, a mutant of Agp1-M15 that carries a chromophore binding Cys at the position of plant phytochromes; and phyB-N651, the N-terminal 651 amino acids of Arabidopsis phyB. All expressed proteins comprise the N-terminal part with PAS-GAF and PHY domains, whereas the C-terminal part is missing. Spectral properties of these fragments are comparable with those of the full-length proteins (Oka et al., 2004; Noack et al., 2007).

Agp1-M15 incorporates BV as a natural chromophore that is covalently bound to Cys20 of the protein; PCB also assembles rapidly with the protein (Lamparter et al., 2002). As in previous studies, the binding mode of PCB is noncovalent: (1) only a very weak Zn²⁺-fluorescence signal was detected in SDS gels (Figure 2C), (2) in the SDS-NAP column assay (see Supplemental Figure 1B online), virtually no chromophore migrated with the protein, and (3) the absorption maximum lies at 690 nm (Figure 2A). Phytochromes that bind PCB covalently have a shorter π electron system and are thus characterized by shorter wavelength absorption maxima around 655 nm. Rapid assembly was also observed for 15-ZaPCB and 15-EaPCB. The 15-ZaPCB spectrum has a maximum at 710 nm (Figure 2A), which is significantly red-shifted versus the absorption maximum of the PCB adduct (690 nm). The absorption peak of the 15-ZaPCB adduct is sharper than that of the PCB adduct. A similar characteristic has been found for 15-ZaBV adducts, which have sharper absorption peaks than the BV adducts (Inomata et al., 2005). The absorption maximum of the 15-EaPCB adducts lies at 735 nm (Figure 2B) and matches well with the Pfr absorption maximum of Agp1-M15-PCB, which lies at 730 nm. According to the long wavelength absorption maxima and the Zn²⁺-fluorescence assay (Figure 2C), both locked chromophores are noncovalently bound to Agp1-M15, although the SDS-NAP column assay shows that chromophore might be tightly bound to the protein (see Supplemental Figures 1C and 1D online). Red-light irradiation of both locked chromophore adducts did not result in any detectable spectral changes (see Supplemental Figure 2A online).

In the Agp1-M15CV double mutant, Cys20 of Agp1 is replaced by Ala, and Val249 is replaced by Cys. This mutant should bind PCB and its derivatives in a covalent manner, because Cys249 is located at the same position within the chromophore pocket as the chromophore binding Cys residue of plant phytochromes (Lamparter et al., 2002). For PCB and both derivatives, the assembly processes of Agp1-M15CV were accompanied by characteristic spectral changes from longer to shorter wavelengths that are indicative of covalent bond formation, during which the ring A ethylidene side chain is lost (see Supplemental Figures 3B to 3D online). Compared with the Agp1-M15 adducts, the absorption maxima of Agp1-M15-CV-PCB, Agp1-15-ZaPCB, and Agp1-15-EaPCB adducts are shifted by 32 to 40 nm to shorter wavelengths (Figures 2D and 2E). Covalent binding was confirmed by Zn²⁺-fluorescence for PCB- and 15-EaPCB-adducts, whereas the band for the 15-ZaPCB adduct was only weak. In the SDS-NAP column assay (see Supplemental Figures 1F to 1H online), all chromophores migrated with the protein, confirming the tight interaction between proteins and chromophores.

When recombinant phyB-N651 was mixed with BV, a low concentration of the bilin remained bound to the protein after the NAP column separation; the adduct was photoactive (see Supplemental Figure 3H online), in agreement with earlier studies (Jorissen et al., 2002). Assemblies with PCB and locked chromophores were again characterized by spectral changes indicative of covalent bond formation. With PCB, assembly-associated spectral changes were completed after ~90 min (see Supplemental Figure 3E online). Covalent linkage was again confirmed by Zn²⁺-fluorescence (Figure 2I) and a NAP-column assay (see Supplemental Figure 1J online). The absorption maximum of the final phyB-N651-PCB adduct was at 650 nm, and the adduct photoconverted into Pfr with a maximum at 709 nm (Figures 2G and 2H). With 15-ZaPCB, assembly-associated

Figure 1. Chromophores Used in This Study.
BV and PCB are the endogenous chromophores. 15-ZaPCB and 15-EaPCB are synthesized chromophores, in which the double bond between C15 and C16 is fixed either in a Z or E configuration.
absorption changes were completed after ~20 min (see Supplemental Figure 3F online). The adduct spectrum resembled the Pr spectrum of phyB-N651-PCB. Compared with the PCB adduct, the peak was again sharper, higher, and red-shifted, in this case by 20 nm (Figure 2G). The assembly with 15\textsuperscript{Ea}PCB was also completed after ~20 min (see Supplemental Figure 3G online). This adduct had an absorption maximum at 715 nm, which is 6 nm red-shifted as compared with the calculated Pfr maximum of the corresponding PCB adduct (Figure 2H). The spectral similarities between the 15\textsuperscript{Za}PCB and 15\textsuperscript{Ea}PCB adducts and the Pr and Pfr forms of phyB-PCB, respectively, suggest that the locked chromophores impose a Pr and Pfr conformation of the protein, respectively.

**In Vivo Studies: C. purpureus Moss Filaments**

In the next steps, we tested the effect of locked chromophores on C. purpureus filaments. It has been found that red light mediated by phytochrome induces chlorophyll synthesis in Ceratodon (Lamparter et al., 1997a). Initially, we used wt4 wild-type cells and the aphototropic mutant ptr116 for feeding studies.

The mutation of ptr116 resulted in a stop codon at position 31 of the heme oxygenase coding sequence, the cause for the chromophore deficiency of that mutant (Brücker et al., 2005). We incubated dark-grown filaments on medium containing BV, PCB, PEB, 15\textsuperscript{Ea}PCB, or 15\textsuperscript{Za}PCB and assayed chlorophyll contents in the tip cell by confocal laser scanning microscopy as in previous studies (Lamparter et al., 1997a). Untreated wild-type wt4 filaments were taken as a control. During the final 24 h, filaments were either kept in darkness or irradiated continuously with red light. Red light increased the chlorophyll contents in wt4 but not in ptr116 tip cells. In the presence of BV or PCB, red light did increase the chlorophyll contents in ptr116 tip cells (Figure 3), in accordance with our earlier study (Lamparter et al., 1997a). Feeding with 15\textsuperscript{Ea}PCB led to an increase of chlorophyll contents in darkness to values in the range of red light–treated ptr116 cells grown on PCB or BV (Figure 3). No differences were found between the 15\textsuperscript{Ea}PCB light- and dark-treated samples. Chlorophyll contents were also slightly increased by PEB and 15\textsuperscript{Za}PCB, which could be because of a feedback effect (Terry and Kendrick, 1999). From these results, we conclude that 15\textsuperscript{Ea}PCB results in the formation of active Pfr in the cell, which...
in turn induces chlorophyll synthesis in darkness. The strong effect of 15EaPCB on chlorophyll synthesis shows that in *C. purpureus* and most likely also in other mosses, all enzymes of the chlorophyll biosynthesis pathway are active in darkness.

Subsequent experiments were performed with a newly isolated wild-type strain, termed K1. When protonemata were incubated on medium with 15EaPCB, chlorophyll contents were increased over the untreated control in darkness (Figure 4), indicating that 15EaPCB induces phytochrome effects also in wild-type cells. It is expected that 15EaPCB competes with the natural chromophore PFB for incorporation into holophytochrome. In earlier experiments (Böse et al., 2004), wt4 and ptr116 were fed with PEB, which is also incorporated into phytochromes and produces fluorescent adducts. In this study, the PEB chromophore was incorporated into approximately one-fourth of phytochrome molecules of wild-type cells, and we expected that approximately the same fraction of phytochrome assemblies with locked chromophore in vivo. The relative increase of chlorophyll contents by 15EaPCB in the K1 wild type was not as strong as the 15EaPCB induction of ptr116 or the induction by red light (Figure 4). This is most certainly the result of partial 15EaPCB incorporation into phytochrome, caused by competition with the internal chromophore. Quite interestingly, the 15EaPCB-induced increase was approximately one-fourth of the red light-induced increase, correlating with the ~25% PEB-chromophore incorporation into wild-type cells mentioned above.

It has been shown that the formation of side branches is induced by phytochromes (Kagawa et al., 1997). We thus tested the effect of 15EaPCB on branch formation (Figure 5). In these experiments, 6% of untreated dark-grown filaments showed

Figure 3. Effect of Light and Chromophores on Chlorophyll Fluorescence of *C. purpureus* Tip Cells in Wild-Type wt4 and the Chromophore Mutant ptr116.

(A) to (F) Fluorescence images of typical tip cells recorded with the same parameter settings.

(A) Dark-grown wild-type wt4.

(B) Red light-irradiated wt4.

(C) Dark-grown ptr116.

(D) Red light-irradiated ptr116.

(E) Red light-irradiated ptr116 on BV.

(F) Dark-grown ptr116 on 15EaPCB.

(G) Relative chlorophyll fluorescence of tip cells. Moss strains, chromophore treatments and light treatments are indicated under each bar. Mean values of 30 or more cells from three experiments ± se. Asterisks denote Student’s t test significance; **, P < 0.01. D, darkness; R, red. Bar in (A) = 20 µm.

[See online article for color version of this figure.]
branching within the uppermost five cells. This fraction increased to 100% in red light (Figure 5). Upon treatment with 15EaPCB, side branches were found in 26% of dark-grown filaments. As above, the increase correlates roughly with the proposed incorporation rate of 15EaPCB.

The gravitropic response of Ceratodon filaments growing in darkness is inhibited by red light via phytochrome (Lamparter et al., 1998; Esch et al., 1999). In our present experiments with K1, in which the filaments were reoriented by 90° and analyzed 48 h later, the tip cells of dark-grown filaments were aligned according to the new growth direction. When these filaments were irradiated with red light after reorientation, the final growth direction was random (Figure 6B). This effect is in accordance with earlier studies that had been performed with wt4 (Lamparter et al., 1998). It should be noted that in the setting of the experiment, the phototropic response is impaired, because irradiation occurs through the cellophane on which the filaments grow. We quantified the curvatures of dark-grown filaments that were either kept on standard medium or exposed to medium with 15ZaPCB or 15EaPCB, whereas the curvatures of filaments grown in red light were not further quantified. The curvature measured at the position of the tip was almost 90° for untreated dark-grown filaments and for filaments grown on 15ZaPCB and 15EaPCB. However, the curvature of these filaments measured 200 μm behind the tip was only 45° in the case of 15EaPCB-treated filaments, as compared with almost 90° in the case of untreated filaments or filaments grown on 15ZaPCB (Figure 6F). This shows that after reorientation, the tip cells find their new

Figure 4. Effect of Light and 15EaPCB on Chlorophyll Fluorescence of C. purpureus K1 Tip Cells.
(A) to (C) Fluorescence images of typical tip cells.
(A) Dark-grown.
(B) Red light–treated.
(C) Dark-grown cell on 15EaPCB.
(D) Quantification of relative chlorophyll fluorescence, mean values of 30 or more tip cells from three experiments ± se. Asterisks denote Student’s t test significance versus dark control; *, P < 0.05; **, P < 0.01.
Bar in (A) = 10 μm.
[See online article for color version of this figure.]

Figure 5. Effect of Light and 15EaPCB on Branch Formation of C. purpureus K1.
(A) Dark-grown filaments.
(B) Red light–treated filaments.
(C) Dark-grown filaments on 15EaPCB.
(D) Calculated branch formation rates, mean values of 30 or more filaments from three experiments ± se. Asterisks denote Student’s t test significance versus dark control; *, P < 0.05; **, P < 0.01.
Bar in (A) = 50 μm.
growth direction later when 15EaPCB is present in the medium and that 15EaPCB specifically reduces the sensitivity of the gravitropism response, most likely via incorporation into phytochrome and formation of Pfr.

In Vivo Studies: Fern Spore Germination

Spore germination of ferns is another phytochrome response that can be monitored at the cellular level (Haupt, 1992; Furuya, et al., 1997). Here, we tested for the effect of 15EaPCB on Adiantum venustum spore germination. The dark germination rate was in the range of 2%, and feeding of 15EaPCB resulted in a spore germination rate of 5%, whereas 49% of the spores germinated upon red-light irradiation (Figure 7D). The 15EaPCB-treated and control samples were different, with an error probability of 10%. In the 15EaPCB-treated spores, only the tip emerged out of the spore envelope, whereas protonemata were clearly visible after red light treatment, indicating that germination was induced after red light treatments. The weak induction could indicate that (1) chromophore uptake through the spore envelope is inefficient, (2) 15Ea-PCB assembly is inefficient, or (3) the 15Ea-PCB adduct is not physiologically active.

In Vivo Studies: Arabidopsis

Most Arabidopsis experiments were performed with the ecotype Columbia (Col) wild type, and experiments with the chromophore-deficient hy1 mutant (Muramoto et al., 1999) were performed with the ecotype Landsberg erecta (Ler) background. In selected cases, we also performed control experiments in which responses of Ler and Col wild types were compared.

We initially tested the effect of chromophores and light on seed germination. Under our test conditions, the wild-type dark germination rate was 2%; this rate increased with red light treatment to almost 100%. Feeding wild-type seeds with BV did not change this dark/light pattern. With 15ZaPCB, the wild-type germination rate was the same as in the dark control, whereas with 15EaPCB, the germination rate was increased to 18% (Figure 8).

The mean dark germination rate of the hy1 mutant was 18% and was thus significantly higher than that of the wild type. Variations of dark germination rates have been reported for many Arabidopsis wild-type and mutant seeds (Koorneef et al., 1985; Poppe and Schäfer, 1997). Red light alone did not increase germination rates of hy1, but when red light was given to BV-treated seeds, germination rates increased to ~60%. BV alone did not stimulate germination in darkness. Feeding with 15ZaPCB did not result in an increased dark germination, whereas 15EaPCB increased germination to ~40% (Figure 8).

Red light inhibits hypocotyl elongation through phytochrome. Under our test conditions, the mean length of Arabidopsis wild-type hypocotyls was 11.1 mm in the dark and 6.5 mm in red light. Again, the 15ZaPCB chromophore had no effect, whereas feeding with 15EaPCB resulted in a significant reduction to 10.0 mm

Figure 6. Effect of Light and Chromophores on the Gravitropic Response of C. purpureus K1.

(A) Dark-grown filaments.
(B) Red light-irradiated filaments.
(C) Dark-grown filaments on 15ZaPCB.
(D) Dark-grown filaments on 15EaPCB.
(E) Tip-to-base curvature of dark-grown filaments without chromophore or with 15ZaPCB or 15EaPCB.
(F) As in (E), curvature between the filament 200 μm behind the tip and the base.

(E) and (F) Mean values of 30 or more filaments from three experiments ± se. The difference in (F) between 15EaPCB-treated filaments and the control is significant: **, P < 0.01.
Bar in (A) = 100 μm.
Hypocotyls of dark-grown hy1 had a mean length of 12.0 mm; red light irradiation did not result in a significant reduction of the hypocotyl lengths. When the mutant was grown on BV, red light reduced the hypocotyl to 6.2 mm. Feeding of 15ZaPCB did not change the hypocotyl length of hy1, whereas growth on 15EaPCB reduced the hypocotyl significantly to 10.6 mm. Thus, 15EaPCB feeding induces a Pfr response in both mutants, but again not to the same extent as BV and red light. We found no differences in hypocotyl lengths under different conditions between the wild types of the Col and Ler ecotype (see Supplemental Figure 4 online).

A similar pattern of chromophore induction was found for cotyledon opening. Under our test conditions, the fraction of wild-type seedlings with open cotyledons was close to zero in darkness. Upon red light irradiation, this fraction increased to 52%. With 15EaPCB (in darkness), 21% of the seedlings had open cotyledons, whereas no induction was found with 15ZaPCB (Figure 9D). In the hy1 mutants, red light alone did not result in a significant induction of cotyledon opening, whereas red light and BV together resulted in 65% of seedlings with open cotyledons; the mean cotyledon opening was increased insignificantly by 15ZaPCB. Feeding with 15EaPCB resulted in 33% of seedlings with open cotyledons.

Phytochromes act as dimers. The induction of phytochrome responses by 15EaPCB in wild-type Arabidopsis suggests that these responses might be induced by heterodimers, in which one subunit carries natural PfrB, and the other subunit carries 15EaPCB. It has been argued that the VLFRs are induced by Pr/Pfr heterodimers, because it is very unlikely that Pfr/Pfr homo-dimers are formed by the weak light sufficient to induce VLFRs (Poppe et al., 1996). Irradiation of Arabidopsis seedlings results in a randomization of the gravitropic response. This effect is...
mediated through phyA and phyB, which induce VLFRs and LFRs, respectively (Liscum and Hangarter, 1993; Poppe et al., 1996; Robson and Smith, 1996). To find out whether 15\textsubscript{Ea}PCB can induce this VLFR, we compared chromophore-treated seedlings with far-red-light–treated seedlings. The results are summarized in Figure 10 as histograms. In darkness, 93% of wild-type seedlings had a vertical growth direction (here defined as 0° to 20° deviation from the vertical). Far-red light treatment stimulated a more random growth direction and reduced the frequency of this group to 64%. BV feeding and feeding with 15\textsubscript{Za}PCB (here darkness only) had no effect on the gravitropic response of the wild type. Feeding with 15\textsubscript{Ea}PCB resulted in a randomization of gravitropism to an extent comparable with the far-red-light–irradiated control. Thus, the VLFR response is completely induced by 15\textsubscript{Ea}PCB.

Dark-grown hy1 seedlings had a more randomized growth direction as compared with the wild type. This difference is not caused by the different ecotypes, because the Ler wild type was indistinguishable from Col, which was used in most experiments. BV-treated, dark-grown hy1 seedlings also revealed a randomized growth direction. A far-red light treatment of hy1 resulted in a more straight growth direction, indicating that some residual active Pfr was reverted into Pr. We note that the hy1 mutants contain low levels of spectrally active phytochrome, but we presently have no explanation for the residual Pfr. Far-red-light–irradiated BV-treated hy1 seedlings had a randomized growth direction, and the effect of far-red light was even stronger than in the wild type. hy1 seedlings treated with 15\textsubscript{Za}PCB had a growth pattern similar to the untreated control.

We also tested by quantitative real-time RT-PCR how messenger RNA (mRNA) levels of three selected phytochrome-regulated genes are affected by the chromophore treatment. Early light-induced proteins are transiently induced during greening of etiolated seedlings (Rossini et al., 2006), and ELIP2 is regulated

Figure 9. Effect of Light and Chromophores on Arabidopsis Hypocotyl Growth and Cotyledon Opening.
(A) and (B) Dark-grown Arabidopsis Col wild type (A) and hy1 (B) seedlings without chromophore (left) and on 15\textsubscript{Ea}PCB (right).
(C) Hypocotyl lengths.
(D) Percentage of seedlings with open cotyledons. Mean values of three experiments ± se (each experiment with 40 or more seedlings). Asterisks denote Student’s t test significance; *, P < 0.05; **, P < 0.01.
Bar in (A) = 2 mm.
[See online article for color version of this figure.]
by phytochrome. Under our test conditions, the mRNA of *ELIP2* was 1.6-fold increased by red light, and 15EaPCB increased the *ELIP2* mRNA level 1.7-fold (Figure 11A). 15ZaPCB treatment resulted in a slightly decreased mRNA level. The mRNA levels of the phytochrome-regulated *PHOTOSYSTEM II TYPE I CHLOROPHYLL A/B BINDING PROTEIN* (*LHB1B2*) (Leivar et al., 2009) were increased 2.2 \( \times 10^3 \)-fold by red light and 13-fold by 15EaPCB (Figure 11B). Again, the expression level was slightly decreased by 15ZaPCB. As an example of a gene whose expression is repressed by light via phytochrome, we chose *PHYTOCHROME INTERACTING FACTOR 3-LIKE1* (*PIL1*) (Hwang and Quail, 2008). This mRNA was downregulated by red light to 6% of the dark control. Feeding with 15EaPCB resulted in a downregulation to 70% of the control (Figure 11C). Feeding with 15ZaPCB showed a slightly increased gene expression level.

**DISCUSSION**

Locked BV-derived chromophores have contributed to the understanding of chromophore stereochemistry in the Pr and Pfr forms. These studies also showed that bacteriopterinchromophores can adopt light-stable Pr and Pfr forms if the appropriate 15Za and 15Ea chromophores, respectively, are incorporated. For experiments with plant phytochromes, the BV derivatives are inappropriate, because these phytochromes incorporate P\(\Phi\)B as a natural chromophore. Although BV can bind to plant phytochromes, the binding mode is noncovalent, and the spectral properties of the adducts are significantly different from natural phytochromes (see Supplemental Figure 3H online) (Quest and Gärtner, 2004). Besides the natural chromophore P\(\Phi\)B, plant phytochromes also incorporate PCB, which has the same ring A ethylidene side chain, in a covalent manner (Figures 2G to 2I; see Supplemental Figure 3E online). We therefore expected that the new chromophores 15ZaPCB and 15EaPCB would also be incorporated in a covalent manner. Indeed, 15ZaPCB and 15EaPCB assembled with Agp1-M15, Agp1-M15CV, and truncated phyB from *Arabidopsis*; the binding mode was noncovalent for Agp1-M15 and covalent for Agp1-M15CV and phyB (Figure 2; see Supplemental Figures 1 and 3 online). Covalent bond formation could be followed by spectral transitions from longer to shorter wavelength absorbance maxima. The spectra of the 15ZaPCB and 15EaPCB adducts were always comparable with those of the Pr and Pfr forms of the respective PCB adduct, and the locked chromophore adducts were photoinactive (see Supplemental Figure 2 online).

We used the moss *C. purpureus* as a first-model plant for the in vivo action of locked chromophores. Feeding of 15EaPCB resulted in an increase of chlorophyll in tip cells of dark-grown protonemata of the chromophore-deficient mutant *prt116* (Figure 3). The increase had the same magnitude as that of the BV-treated mutant in red light. We conclude from these observations that (1) locked chromophores are efficiently taken up by the tip cells, like other bilin chromophores, (2) the 15Ea-phytochrome adduct is fully active and induces the signal transduction cascade, and (3) in the moss cells, no other input is required for the induction of chlorophyll synthesis. This finding reveals an experimental insight into the evolution of chlorophyll synthesis: one of the latest steps in the biosynthesis pathway is the conversion of protochlorophyllide to chlorophyll, catalyzed by the protochlorophyllide-chlorophyll oxidoreductase. Two versions of protochlorophyllide-chlorophyll oxidoreductase exist, a light-dependent enzyme (LPOR) and a light-independent
enzyme (DPOR). Angiosperms have only LPORs, and chlorophyll synthesis is strictly light-dependent. Other plants and photosynthetic bacteria may have DPOR and LPOR, and it has been shown that gymnosperms, ferns, algae, cyanobacteria, and other bacteria can synthesize chlorophyll in darkness (Fujita, 1996; Reinbothe et al., 2010). The genome of the moss Physcomitrella patens contains two genes that might encode for LPORs and one that might encode for DPOR. Our Ceratodon results show that a DPOR is indeed active in mosses, as proposed earlier (Lamparter et al., 1997a). By using 15EaPCB, one might discriminate between LPOR and DPOR action in the future.

Studies with a new Ceratodon strain showed that 15EaPCB, but not 15ZaPCB, can also induce phytochrome responses in wild-type cells (Figures 4 to 6). We assume that the major fraction of phytochrome in the cell harbors the natural chromophore, and most 15EaPCB chromophores are incorporated into heterodimers in which the other subunit carries PfB and remains in the Pr state. This suggests that the relevant effects might be induced by a mixed Pr/15Ea-adduct heterodimer, although a clear statement with respect to heterodimer/homodimer function cannot be drawn on the basis of the available data.

For studies with Arabidopsis, we made use of chromophore-deficient mutant hy1. This mutant has been investigated by many groups and has led to the identification of an Arabidopsis heme oxygenase gene (Parks and Quail, 1991, Muramoto et al., 1999). The major outcome of our Arabidopsis studies was that all phytochrome responses under investigation can be induced by 15EaPCB in darkness (Figures 8 to 11). Not only the chromophore-deficient mutant, but also wild-type plants showed such an induction. Another finding is equally important: In most Arabidopsis phytochrome effects investigated here, the induction by 15EaPCB in the dark was not as strong as the induction by red light given to the wild-type plant or to a BV-grown hy1 mutant (Figures 8 to 11). The magnitude of the response could be related to subtle structural differences between natural Pfr and the 15EaPCB adducts that result in differential coupling to the signal transduction cascade. We consider however that the induction by 15EaPCB is limited, because the adducts do not undergo cycling through Pr and Pfr. Studies with the Y276H mutant of phyB (Su and Lagarias, 2007) and the corresponding Y242H mutant of phyA (Rausenberger et al., 2011) show that these mutants induce a constitutive deetiolated phenotype when expressed in Arabidopsis in darkness, just like the locked 15EaPCB chromophore. The effect of phyA_Y242H was less pronounced than that of phyB_Y276H, and phyA_Y242H induced a dominant negative effect in the light when overexpressed in wild-type Arabidopsis (Rausenberger et al., 2011). The difference in the action of both phytochrome mutants is explained by a phyA/phyB differential nuclear import mechanism: Maximal uptake of phyA into the nucleus requires cycling through Pr and Pfr, whereas nuclear uptake of phyB does not include cycling (Pfeiffer et al., 2009). According to this model, phyB responses can be maximally induced by 15EaPCB, whereas phyA responses are only partially induced by this chromophore.
We are presently investigating the effect of 15EaPCB on particular phytochrome mutants. The specific properties of phytochrome A have evolved in seed plants (Schneider-Poetsch et al., 1998, Mathews, 2005; Lamparter, 2006; Mathews, 2010), and mosses do not contain a phytochrome A homolog. The finding that chlorophyll synthesis is induced by 15EaPCB in the pti16 mutant of C. purpureus to the same extent as by red light after BV treatment (Figure 3) is consistent with a phyB-like nuclear uptake mechanism or phytochrome action outside the nucleus. It should be noted that microbeam experiments provided evidence that cycling is used by Ceratodon phytochromes to measure very strong light intensities (Lamparter et al., 2004).

A more general conclusion also arises from our studies. The 15Ea chromophores yield Pfr-like adducts, resulting in Pfr-like protein conformations in vitro (Inomata et al., 2005), and also actively induce phytochrome responses in vivo, as shown here. These results support the idea that an isomerization around the C15–C16 double bond is the driving force for the Pr-to-Pfr conversion and argue against the recent hypothesis formulated in the context of NMR data on a phytochrome-like protein from the cyanobacterium SyB-Cph1 (Ulijasz et al., 2010) that ring A motions might be the first step.

METHODS

Chromophores

BV was purchased from Frontier Scientific. PCB was prepared as described before (Kunkel et al., 1995). The locked PCB chromophores 15ZaPCB and 15EaPCB were described recently (Nishiya et al., 2010). Chromophore stock solutions were prepared in DMSO at concentrations of 1 to 10 mM and stored at −80°C. For in vitro assembly studies, the final chromophore concentrations were between 1 to 10 μM, and the molar ratio between protein and chromophore was always 1:1. For in vivo studies, the final chromophore concentration in the growth medium was always 10 μM (Lamparter et al., 1997a). Control medium was prepared with an equal DMSO concentration.

Expression Vectors

All recombinant phytochromes were expressed as apoproteins in Excichia coli. The vector pAG1-M15 (Noack et al., 2007) was used for expression of Agp1-M15, the truncated version of Agp1, lacking the His kinase. A clone for Agp1-M15CV, in which Cys20 is replaced by Ala and Val249 is replaced by Cys, was also used. The mutations were introduced by site-directed mutagenesis according to the protocol for Quik Change Site-Directed Mutagenesis (Stratagene); the primers were CCGAAACTG GATAGTGCCGGGAGGGCCATCC/GGATGGCGCTTGCGCCCGC CACTATCCAGTTTCGG for the conversion of Cys20 to Ala and CCGAGGCTATGAGCGGTGTCATCTCGAAATATA and TATATTCGAGATGAG CACGCGGATACGCTGCG for the conversion of Val249 to Cys. For cloning of the N-terminal chromophore module of Arabidopsis thaliana phytochrome B (phyB-N651), the coding sequence was amplified by PCR from genomic DNA of Arabidopsis using Phusion polymerase (Finnzymes). The PCR conditions were annealing at 62.4°C for 30 s, extension at 72°C for 70 s, 35 cycles. The sequence of the primers were ATATGCGTCTGCTCCGTGGCAGGTGGTAG (forward) and GGATGGGCGCCACATCACTACTACTCAGCCGTCCGGT (reverse). The PCR product was digested with Nhel and EcoRI and cloned into the Nhel-EcoRI-digested expression vector Pet21b (Invitrogen). The resulting open reading frame begins with the original start codon and ends with six additional His codons. The insert was sequenced and proved identical with the sequence from the database.

Expression and Purification of Recombinant Phytochromes

Agp1-M15 and Agp1-M15CV were expressed and purified as before (Lamparter et al., 1997b; Noack et al., 2007). For recombinant phyB-N651, the expression clones were initially grown overnight in liquid 4 × 5 mL rich broth at 37°C. The entire volume was mixed with 4 × 1 L rich broth medium, and E. coli was propagated at 37°C until A600 nm reached 0.5 (after −3 h). Expression of phytochrome was induced by the addition of 25 μM isopropyl-1-thio-β-d-galactopyranoside. Thereafter, cultures were grown for −22 h at 14°C until A600 nm reached 2.0. Cells were then harvested by centrifugation. The cell pellet was washed twice with 200 mL extraction buffer (20 mM Tris/Cl, 5 mM EDTA, pH 7.8), suspended in 40 mL extraction buffer, and passed through a French pressure cell (American Instrument Company) at 10,000 psi for 1 min. The supernatant of a 30-min centrifugation at 20,000g was precipitated by 50% saturated ammonium sulfate. After another 30-min centrifugation at 20,000g, the pellet was suspended in 20 mL wash buffer (25 mM imidazole, 500 mM NaCl, 20 mM Tris/Cl, pH 7.8) and centrifuged again. The supernatant was loaded to a 2.5 × 1.7 cm Ni2+-NTA (Qiagen) affinity column. The column was washed with 4 L wash buffer, and finally the protein was eluted with 100 mM imidazole, 500 mM NaCl, 20 mM Tris/Cl, pH 7.8. Fractions with A280 nm > 0.3 were collected, and protein was precipitated again with 50% saturated ammonium sulfate. The protein pellet was dissolved in 2 mL extraction buffer (20 mM Tris/Cl, 500 mM NaCl, 5 mM EDTA, pH 7.8) with 1 mM tris(2-carboxyethyl)phosphine and stored at −80°C.

Assembly, UV/Visible Spectroscopy, NAP Column Separation

For assembly and photoconversion assays, the samples were kept at 18° C. Absorption spectra were measured in a JASCO V-550 photometer with a custom-built computer-controlled irradiation device. The scan speed was set to 1000 nm min−1; scans were measured between 900 and 250 nm. To monitor chromophore assembly, a 1 to 10 μM recombinant protein solution was prepared to which DTT was added to a final concentration of 2 mM. Chromophore from DMSO stock solution was added to the same final molar concentration as that of the apoprotein. The sample was mixed rapidly, and absorption spectra were recorded immediately and at different time points until no more absorption changes were apparent. Spectra of free chromophores in buffer solution were recorded in parallel. The solution was passed over a NAP-10 column (GE Healthcare) to separate the protein from free chromophore. Details on chromatophore assembly and NAP column separation can be found in Lamparter et al. (2002) and Inomata et al. (2006).

For photoconversion, the samples were irradiated in the cuvette with light-emitting diodes of 655 nm (red, 510 μmol m−2 s−1), 730 nm (far-red, 440 μmol m−2 s−1), or 780 nm (long wavelength far-red, 1000 μmol m−2 s−1) for 2, 4, and 4 min, respectively. After irradiation with saturating red light, the Pfr/total phytochrome concentration (Ptot) ratio of a typical phytochrome lies between 0.6 and 0.9, depending on its specific biochemical properties; pure Pfr spectra can only be measured in rare cases (e.g., bathyphytochromes). To allow spectral comparisons of Pr and Pfr with 15EaPCB and 15ZaPCB adducts, we estimated pure Pfr spectra by an empirical approach (Inomata et al., 2005), which also gives a rough Pfr/Ptot ratio. To this end, we use the formula

\[
A_{\text{Pr}} = \left( A_{\text{Pr}, \text{meas}} - A_{\text{Pr}, \text{raw}} \times (1 - c) / c \right)
\]

where wavelength (λ)-dependent absorbance values of pure Pfr, of the sample after red irradiation, and of Pr (dark sample) are given by \( A_{\text{Pr}, \text{meas}}, A_{\text{Pr}, \text{meas}}, \) and \( A_{\text{Pr}, \text{meas}}, P_{\text{Pfr}}, P_{\text{Ptot}} \) is given by c. Calculations of Pfr spectra start with a c value of, for
example, 0.7; this parameter value is sequentially increased or decreased until calculated spectra have a PfR-like appearance. This final c value and the calculated PfR spectrum are given in Figure 2. According to our experience, the precision of c is better than ±0.1.

SDS-PAGE and Zn²⁺ Fluorescence

Covalent chromophore binding was assayed by Zn²⁺ fluorescence (Berkelman and Lagarias, 1986). For these assays, chromophore assembly was performed for 5 h, and the NAP column separation was omitted. SDS-PAGE was performed under standard conditions (Laemmli, 1970), using 3% stacking gels and 10% resolving gel. Before electrophoresis, 1 mM Zn²⁺-acetate was added to the running buffer. After electrophoresis, the gels with fluorescent biliprotein bands were photographed using a UV transilluminator, and the protein bands were stained with Coomassie blue (Sambrook and Russell, 2001). SDS-NAP column assays were performed in the presence of 1% SDS in adduct samples and elution buffer. Spectra before and after column separation were compared; for presentation, absorbance values are normalized to A₂₆₀nm.

Plant Material and Growth Conditions

*Moss Ceratodon purpureus*

The *C. purpureus* wild-type wt4 (Hartmann et al., 1983) and the phototropic mutant ptr116 (Lamparter et al., 1997a) derived from the same strain were used in this study. The filaments of the wild-type wt4 and the mutant ptr116 have been grown for more than 20 years in vegetative cultures. The growth rates of these filaments are slower than before, and physiological responses are impaired. For this reason, we also used a new *C. purpureus* wild type that has been isolated from fresh gametophyte tissue. This new strain is termed K1. The moss plants were kindly provided by Dr. A. Hölzer (Naturkundemuseum Karlsruhe). Protothæmata were grown on medium 1b (Lamparter et al., 1997a) derived from the same strain were used in this study. The physiological responses are impaired. For this reason, we also used a new *C. purpureus* wild-type strain that has been isolated from fresh gametophyte tissue. This new strain is termed K1. The moss plants were kindly provided by Dr. A. Hölzer (Naturkundemuseum Karlsruhe). Protothæmata were grown under 16 h/8 h light/dark cycles at 25°C on medium 1b (Lamparter et al., 1997a) with the following modifications: moss filaments were grown for 3 d in darkness on vertical Petri dishes covered with 1 cm × 1 cm cellophane pieces and then transferred to new vertical plates containing 10 μM bilin. After the transfer, the moss filaments were repositioned so that the vertically aligned filaments became horizontally oriented. Thereafter, filaments were cultivated for another 48 h either in darkness or in red light (660 nm, 4 μmol m⁻² s⁻¹, irradiated through the agar medium). Finally, images were taken using a Leica DFC500 camera, connected to a Leica M420 microscope. For side branch formation, the apical five cells of the filaments were inspected. The angle of gravitropic curvature was either measured between the tip and the base of each filament or between 200 μm behind the tip and the base.

Chlorophyll Fluorescence Assays

Chlorophyll quantification in single tip cells was performed as described earlier (Lamparter et al., 1997a) with the following modifications: moss filaments were grown for 7 d (wt4, ptr116) or 3 d (K1) in darkness on 1 cm × 1 cm cellophane pieces, which were positioned on vertically oriented agar plates, then transferred to agar with or without bilin and cultivated for another 24 h. Some filaments were irradiated with red light from an array of 660-nm light-emitting diodes; the light intensity was 4 μmol m⁻² s⁻¹. Chlorophyll contents of tip cells were estimated by fluorescence using a confocal laser scanning microscope (TCS SP1; Leica) with a Krypton Argon laser. The wavelength for exciting light was set to 633 nm, and the emission was recorded through a 670-nm band pass filter. This setup is specific for chlorophyll, and precursors such as protochlorophyllide, which has an emission maximum at 631 nm, would not be detected (Mattheis and Rebiez, 1975; Hukmani and Tripathy, 1992).

Apical cells were selected arbitrarily through the normal optics of the microscope. For all measuring procedures, the high voltage of the photomultiplier was set to 1000 V, and the pinhole was set to 1.0. The light intensity was always set to 20%, to generate signals just below the saturation level, and the signals of four frames were averaged. For each specimen, fluorescence images of 10 or more cells were stored on hard disk. For each cell, the fluorescence intensity within the apical 150 μm of the tip cell was estimated using the imaging software ImageJ. Background noise was subtracted. The laser has no direct effect on chlorophyll levels: In control experiments in which the cells were irradiated for 30 min with the laser of the microscope, we found no significant change of chlorophyll fluorescence.

Branch Formation and Gravitropism Response

For branch formation and gravitropism experiments, K1 filaments were first grown for 3 d in darkness on vertical Petri dishes covered with 1 cm × 1 cm cellophane pieces and then transferred to new vertical plates containing 10 μM bilin. After the transfer, the moss filaments were repositioned so that the vertically aligned filaments became horizontally oriented. Thereafter, filaments were cultivated for another 48 h either in darkness or in red light (660 nm, 4 μmol m⁻² s⁻¹, irradiated through the agar medium). Finally, images were taken using a Leica DFC500 camera, connected to a Leica M420 microscope. For side branch formation, the apical five cells of the filaments were inspected. The angle of gravitropic curvature was either measured between the tip and the base of each filament or between 200 μm behind the tip and the base.

Adiantum venestum Spore Germination

Spores of *A. venestum* were collected in the Botanical Garden of the Karlsruhe Institute of Technology (KIT) and stored at room temperature in plastic vials. For each germination experiment, spores were scrapped off the wall of the vial using a pipette tip. The spores were mixed with 2 mL one-tenth-strength Murashige and Skoog medium (Murashige and Skoog, 1962) containing 0.08% agar and were poured into Petri dishes of 2 cm diameter. After 7 d incubation at 25°C, spore germination was checked under the microscope. For the 15EaPCB treatment, the chromophore was added to fresh medium to reach a final concentration of 20 μM. Fresh chromophore at the same concentration was added on the subsequent day and on the third day. For red-light treatment, spores were irradiated during the first 3 d with the 660-nm light-emitting diode; the light intensity was 66 μmol m⁻² s⁻¹.

Arabidopsis Seed Germination

The *hy1* mutant (Lar ecotype) was obtained from the Nottingham Arabidopsis Stock Centre and propagated in the greenhouse of the Botanical Garden. Seeds were collected and stored at 4°C until use. Wild-type seeds (Col and Ler ecotype) were kindly provided by members of the Botanical Garden (KIT Karlsruhe). Seeds were surface-sterilized for 1 min in 70% ethyl alcohol and for 7 min in 1:1 (v/v) sodium hypochlorite, rinsed four times with sterile H₂O, then planted on cellophane-overlaid Petri dishes containing one-half-strength Murashige and Skoog salts (Murashige and Skoog, 1962), 0.05% MES, and 0.8% (w/v) agar without Suc. The plates were incubated for 2 d at 4°C in darkness. Thereafter, the seeds were transferred with the cellophane to fresh plates containing 10 μM bilin or no bilin and then either irradiated with red light (660 nm, 66 μmol m⁻² s⁻¹) for 5 min and transferred back to darkness or continuously kept in darkness. Germination was checked after 2 d. All manipulation steps were performed under green dim safelight.

Arabidopsis Hypocotyl Length and Cotyledon Unfolding

These experiments were performed with *hy1* mutants and the wild type of the Col and Ler ecotypes. Seeds were sterilized as above and incubated on cellophane-overlaid agar plates and kept in darkness for 4 d at 4°C. The plates were then placed under fluorescent light tubes (OSRAM L 360W/765) for 4 h to induce uniform germination; the light intensity was 90 μmol m⁻² s⁻¹. The light-treated seeds were incubated in darkness at
25°C for an additional 48 h. Thereafter, the cellophane with the germinated seedlings was transferred to new agar medium containing 10 μM bilin and then directly placed in red light (66 μmol m\(^{-2}\) s\(^{-1}\)) or darkness. The agar plates were always vertically oriented so that the seedlings remained attached to the surface of the medium. In this way, bilin uptake was optimized. After 2 d of growth, the seedlings were photographed, and growth parameters were quantified using ImageJ.

**Arabidopsis Gravitropism**

Seeds were surface-sterilized as above, kept at 4°C for 2 d in darkness, then illuminated for 2 h with white light to induce germination and were incubated in darkness for 22 h. Thereafter, the seedlings were transferred to new plates with or without chromophore and kept for an additional 2 d at 25°C; the plates were vertically oriented. The VLFR response was induced by far-red light (730-nm light-emitting diodes, 10 μmol m\(^{-2}\) s\(^{-1}\)) given for 5 min per h from above during the first 12 h. After this treatment, images were taken, and the hypocotyl angles with respect to the vertical were measured using ImageJ. An upward growth direction is defined as 0°. For data presentation, five groups were distinguished: 0° to 20°, 20° to 40°, 40° to 60°, 60° to 80°, and 80° to 90°.

**Arabidopsis Quantitative Real-Time RT-PCR**

After uniform seed germination as above and 3 d dark-growth on vertical plates, seedlings were transferred to fresh medium containing 15 μM bilin and then directly placed in red light (66 μmol m\(^{-2}\) s\(^{-1}\)) for 5 min from above during the first 12 h. After this treatment, images were taken, and the hypocotyl angles with respect to the vertical were measured using ImageJ. An upward growth direction is defined as 0°. For data presentation, five groups were distinguished: 0° to 20°, 20° to 40°, 40° to 60°, 60° to 80°, and 80° to 90°.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure 1.** SDS-NAP Column Assays with Phytochromes.

**Supplemental Figure 2.** Photoconversion of (Locked) Chromophore Adducts.

**Supplemental Figure 3.** Phytochrome Assembly; Photoconversion of phyB-N651-BV.

**Supplemental Figure 4.** Effect of Light and Chromophores on Arabidopsis Hypocotyl Elongation and Gravitropic Response, Comparisons between Ler and Col Ecotypes.

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**AUTHOR CONTRIBUTIONS**

K.N., A.K., Y.U., and K.I. synthesized the locked chromophores, R.Y. performed the other experiments, R.Y., K.I., and T.L. wrote the article, R.Y., K.I., and T.L. designed the study, and T.L. supervised the study.

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Assembly of Synthetic Locked Phycocyanobilin Derivatives with Phytochrome in Vitro and in Vivo in Ceratodon purpureus and Arabidopsis

Rui Yang, Kaori Nishiyama, Ayumi Kamiya, Yutaka Ukaji, Katsuhiko Inomata and Tilman Lamparter

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