Low Blue Light Enhances Phototropism by Releasing Cryptochrome1-Mediated Inhibition of *PIF4* Expression[1[OPEN]]

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Shade-avoiding plants, including Arabidopsis (*Arabidopsis thaliana*), display a number of growth responses, such as elongation of stem-like structures and repositioning of leaves, elicited by shade cues, including a reduction in the blue and red portions of the solar spectrum and a low-red to far-red ratio. Shade also promotes phototropism of de-etiolated seedlings through repression of phytochrome B, presumably to enhance capture of unfiltered sunlight. Here we show that both low blue light and a low-red to far-red light ratio are required to rapidly enhance phototropism in Arabidopsis seedlings. However, prolonged low blue light treatments are sufficient to promote phototropism through reduced cryptochrome1 (cry1) activation. The enhanced phototropic response of *cry1* mutants in the lab and in response to natural canopies depends on *PHYTOCHROME INTERACTING FACTORs* (*PIFs*). In favorable light conditions, cry1 limits the expression of *PIF4*, while in low blue light, PIF4 expression increases, which contributes to phototropic enhancement. The analysis of quantitative DII-Venus, an auxin signaling reporter, indicates that low blue light leads to enhanced auxin signaling in the hypocotyl and, upon phototropic stimulation, a steeper auxin signaling gradient across the hypocotyl. We conclude that phototropic enhancement by canopy shade results from the combined activities of phytochrome B and cry1 that converge on PIF regulation.

In natural environments, light conditions are highly dynamic and heterogeneous, and given the importance of light for their survival, plants have evolved sophisticated photosensory systems to integrate multiple light cues (Casal, 2000; Paik and Huq, 2019). The presence of dense vegetation is not well tolerated by sun-loving plants, such as Arabidopsis (*Arabidopsis thaliana*). Plants detect neighbors by sensing the low-red (R) to far-red (FR) ratio (LRFR), which is a consequence of FR reflection by leaves. If the vegetation becomes denser, a canopy filters sunlight, creating an environment with LRFR and reduced blue light, R light, and photosynthetically active radiation (PAR; Fiorucci and Fankhauser, 2017). Enhanced hypocotyl elongation and leaf elevation, reduction of branching, and flowering acceleration are some of the mechanisms that have evolved to optimize light capture and increase fitness in response to vegetational shade (Ballaré and Pierik, 2017).

Natural canopies are not uniform, and gaps allow unfiltered light to create light gradients (Fiorucci and Fankhauser, 2017). Thus, when canopy shade is combined with a directional blue light gradient, plants reorient stem growth to position their photosynthetic organs toward blue light, in a process called phototropism (Ballaré et al., 1992; Fiorucci and Fankhauser, 2017). Phototropism is mainly controlled by the phototropin blue light receptors (phot1 and phot2 in Arabidopsis), which trigger a number of physiological responses (Liscum and Briggs, 1995; Sakai et al., 2001). Blue light activation of phototropins generates an asymmetrical distribution of auxin across the hypocotyl, which leads to asymmetrical cell growth between the shaded and lit sides of the hypocotyl (for review, see Fankhauser and Christie, 2015; Legris and Boccaccini, 2020).

Unlike etiolated seedlings, which show high sensitivity to directional blue light, de-etiolated seedlings growing in full sunlight do not show a strong phototropic response (Goyal et al., 2016; Schumacher et al., 2018). However, LRFR, which is typical of shaded environments, enhances phototropism (Ballaré et al., 1992; Goyal et al., 2016). The inactivation of phytochrome B (phyB) by LRFR leads to the accumulation/activation of

PHYTOCHROME INTERACTING FACTOR4 (PIF4), PIF5, and PIF7, which promote expression of *YUCCA* genes (*YUC2*, *YUC5*, *YUC8*), encoding enzymes for auxin biosynthesis (Hornitschek et al., 2012; Li et al., 2012; Kohnen et al., 2016). This up-regulation of auxin biosynthetic genes in the cotyledons is sufficient for hypocotyl reorientation in LRFR (Goyal et al., 2016).

Phenotypical experiments of seedlings defective for another class of blue light photoreceptors, called cryptochromes (cry), reveal that they modulate phototropism with a positive role in etiolated seedlings (Whippo and Hangarter, 2003; Ohgishi et al., 2004; Tsuchida-Mayama et al., 2010) and a potentially negative role in de-etiolated seedlings (Goyal et al., 2016). The Arabidopsis genome encodes two cry, cry1 and cry2, which coordinate blue light-mediated gene expression by the inactivation of the CONSTITUTIVE PHOTOMORPHOGENIC1/SUPPRESSOR OF PHYA-105 (COP1/SPA) E3 ligase complex (Holtkotte et al., 2017; Lau et al., 2019; Ponnu et al., 2019) or through the interaction with several transcription factors (Liu et al., 2008; Ma et al., 2016; Pedmale et al., 2016; Wang et al., 2018; Xu et al., 2018; He et al., 2019; Mao et al., 2020). Light-induced activation of cry1 and cry2 is controlled by BLUE-LIGHT INHIBITORS OF CRYPTOCHROMES1 (BIC1) and BIC2 (Wang et al., 2016). cry1 and cry2 are associated with chromatin, where they are proposed to control transcription factor activity through incompletely characterized mechanisms (Ma et al., 2016; Pedmale et al., 2016). When expressed in a heterologous system, cry2 interacts with DNA and promotes gene expression in a blue-light-induced manner (Yang et al., 2018).

At the physiological level, cry control several responses, such as promotion of blue-light-induced de-etiolation and photoperiodic flowering (Yang et al., 2017). In conjunction with phyB, cry also controls shade avoidance responses (Millenaar et al., 2009; Pierik et al., 2009; Keller et al., 2011). In low blue light (LBL) conditions, which is one of the features of canopy shade, the activity of cry is reduced to trigger hypocotyl and petiole elongation (Millenaar et al., 2009; Pierik et al., 2009; Keller et al., 2011; de Wit et al., 2016). One of the mechanisms used by cry to exert their activity is through interaction with PIF4 and PIF5 transcription factors and regulation of their activity (Ma et al., 2016; Pedmale et al., 2016). Given the involvement of cry in canopy shade responses and phototropism (Millenaar et al., 2009; Pierik et al., 2009; Keller et al., 2011; Goyal et al., 2016), we examined how cry modulate hypocotyl growth reorientation in response to blue light features of canopy shade. In our conditions, we found that cry1 is the main cryptochrome involved in the attenuation of phototropism in sunlight-mimicking conditions, and cry1-mediated inhibition of *PIF4* expression is a component of this regulation. Our results reinforce the relevance of the cry1-PIF4 module in light-mediated processes. It emerges as a key module not only for the regulation of hypocotyl elongation, but also for the reorientation of hypocotyls to avoid canopy shade.

RESULTS

Persistent LBL Promotes Phototropism

Multiple features of the light environment altered by canopy shade can be mimicked by combining LBL and LRFR (de Wit et al., 2016). In a previous publication, we showed how LRFR enhances phototropism through inactivation of phyB. However, the phenotype of *cry1* suggested that LBL typical of canopy shade also influences hypocotyl reorientation (Goyal et al., 2016). To determine how specific features of canopy shade contribute to enhanced phototropism, we measured hypocotyl curvature in the lab under full white light (WL; high blue light and high R to FR ratio), under LBL (blue light was depleted by covering the seedlings with a yellow filter), under LRFR (FR was added to the WL), or under the combination of both LBL and LRFR to simulate the canopy shade (SCS; Fig. 1A; de Wit et al., 2016). Given that LBL enhances hypocotyl growth more slowly than LRFR (Pedmale et al., 2016), we decided to include treatments with different light qualities 24 h prior to testing their phototropic potential (referred to as pretreatment Fig. 1A, e.g. LRFR/LRFR and LBL/LBL). In all conditions analyzed, the seedlings were exposed to supplementary horizontal blue light (8 μmol $m^{-2}s^{-1}$) during phototropic stimulation (Fig. 1A). We measured deviation from vertical growth after 6 h of lateral blue light treatment. The overall bending of wild-type (Col-0) seedlings in WL/WL, WL/LBL, and WL/LRFR was modest, indicating that neither LBL nor LRFR alone were sufficient to trigger a significant enhancement of hypocotyl curvature (Fig. 1, B and C). However, we observed a nonsignificant tendency for

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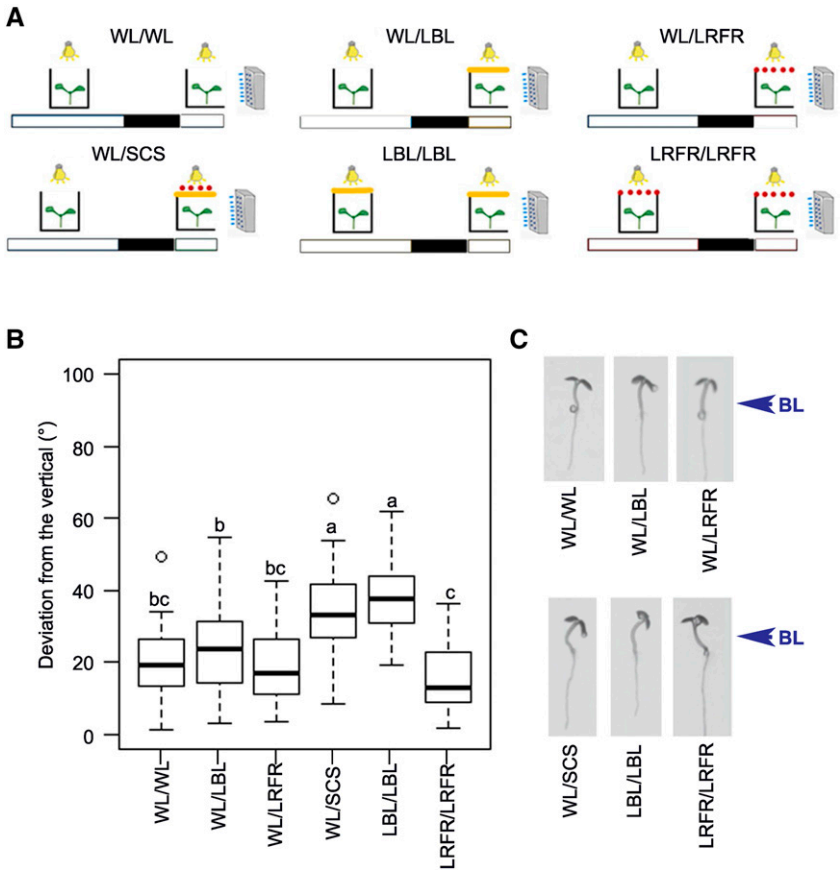

Figure 1. The blue light component of canopy shade is critical for phototropism in green seedlings. A, Experimental scheme, which represents the day preceding the application of lateral blue light and treatment during phototropism. WL, High blue light and high red to far-red ratio; LBL, low blue light and high red to far-red ratio; LRFR, high blue light and low red to far-red ratio; SCS, low blue light and low red to far-red ratio. Bulbs represent the sources of white light, orange lines represent the filters used to lower blue light, red dots represent the sources of FR, and blue dots represent the sources used to provide horizontal blue light. On the day of the phototropic assay, the new light treatment was started a few minutes after ZT0. Refer to "Materials and Methods" for irradiance values. B, Box plots represent the deviation from the vertical of 4-d-old seedlings ($n \geq 25$) 6 h after lateral blue light application. Letters indicate statistically significant differences at $P < 0.05$ obtained by one-way ANOVA followed by the post-hoc Tukey's HSD. C, Representative seedlings of the experiment shown in B.

increased bending in WL/LBL (Fig. 1, B and C). Moreover, when LBL was combined with LRFR (WL/SCS), phototropism was significantly enhanced (Fig. 1, B and C). The LRFR condition described in Goyal et al. (2016), stimulates phototropism; however, here seedlings were grown in long days under stronger WL to more closely mimic a natural environment. Interestingly, expanding LBL exposure to the day before phototropic stimulation (LBL/LBL) significantly enhanced the phototropic response compared to WL/LBL (Fig. 1). In the presence of the same amount of blue light provided unilaterally, the yellow filter used to create the LBL environment changed the blue light differential between the top and the illuminated side. However, this does not appear to be the reason for enhanced bending in LBL/LBL (as WL/LBL does not significantly enhance bending, and see next section) and allowed us to specifically study the effect of LBL on phototropic responsiveness (Fig. 1B, and further experiments below). Remarkably, LBL, but not LRFR, pretreatment affected the phototropic response (Fig. 1, B and C; Supplemental Fig. S1A), although both treatments induced hypocotyl elongation (Supplemental Fig. S1B). Moreover, treatment with a neutral filter to reduce PAR intensity the day before phototropic stimulation did not affect phototropism (Supplemental Fig. S1C). To better define when the LBL pretreatment was most effective to promote phototropism the following day, LBL treatment was started or ended at different times of the first day (Supplemental Fig. S1, D and E). To be effective, the LBL treatment had to begin by Zeitgeber time 9 (ZT9) for a full pretreatment effect and at ZT12 for a significant effect (Supplemental Fig. S1D). In addition, more than 4 h of WL before the end of the day (LBL pretreatment ended at ZT9) fully abolished the pretreatment effect, but 1 h of WL after 15 h of LBL pretreatment barely altered bending the next day (Supplemental Fig. S1E). Therefore, the duration and/or time of day of the previous-day LBL treatment mattered. We conclude that a prolonged reduction of blue light in the environment promotes phototropism and is not merely a consequence of enhanced hypocotyl elongation.

Persistent LBL Relieves the Inhibitory Effect of cry1 on Phototropism

Cry are the photoreceptors sensing blue light reduction in canopy shade (Keller et al., 2011; de Wit et al., 2016; Pedmale et al., 2016), and they also modulate hypocotyl reorientation in etiolated seedlings (Whippo and Hangarter, 2003; Ohgishi et al., 2004; Tsuchida-Mayama et al., 2010). To define cryptochrome function during shade-enhanced phototropism, we compared hypocotyl growth reorientation of the wild type

and *cry1* mutant in response to different WL and LBL (pre-) treatment combinations (Fig. 2, A and B). When phototropism was performed in LBL, 24 h of LBL pretreatment strongly accelerated the phototropic response of wild-type seedlings (Fig. 2A). Remarkably, *cry1* seedlings were insensitive to the high levels of blue light present under WL pretreatment conditions and responded like the LBL-pretreated wild type (Fig. 2A; Supplemental Fig. S2A). Our experiments showed that an LBL pretreatment enhanced phototropism when it was analyzed either in LBL (Fig. 2A) or WL (Fig. 2B) showing that the enhanced response is not due to a change in the blue light gradient. To confirm this, we performed the same experiments in WL conditions but increased the horizontal blue light intensity to match the gradient in LBL (see "Materials and Methods"). Both in wild type and *cry1*, we did not detect significant differences between the WL responses in high versus low gradient (Supplemental Fig. S2B). In addition, the increased gradient in WL never led to the phenotype observed in LBL/LBL (Supplemental Fig. S2B). Lastly, in our light conditions, only *cry1* and *cry1cry2*, but not *cry2*, exhibited a de-repressed phototropic response similar to LBL-pretreated wild-type seedlings (Fig. 2C). Taken together, our experiments indicate that cry1 suppresses the phototropic response in WL conditions and reduced cry1 activation in LBL releases this suppression.

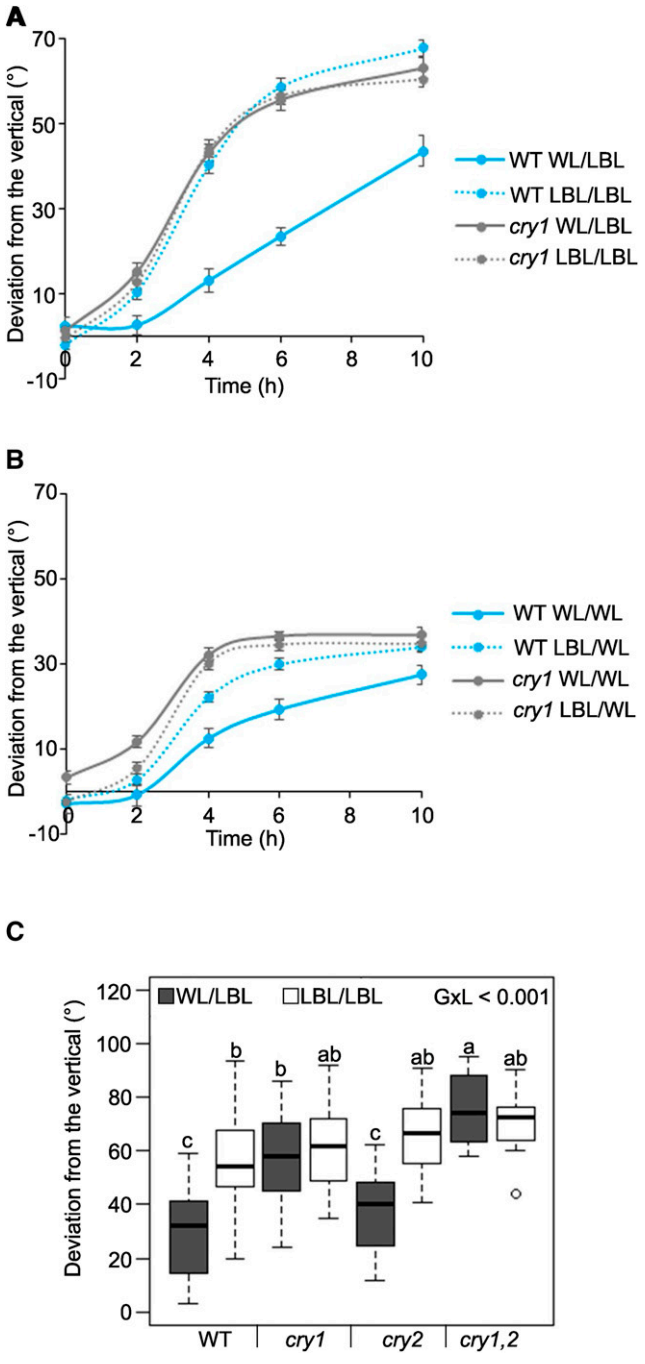

Figure 2. Persistent LBL relieves the inhibitory effect of cry1 on phototropism. Time course analysis of hypocotyl curvature in wild-type (WT) and *cry1* 3-d-old seedlings in LBL (A) or in WL (B) with or without LBL pretreatment. Values represent means ($n \geq 25$) ± SE. C, Box plots represent the deviation from the vertical of 3-d-old seedlings ($n \geq 25$) 6 h after lateral blue light application. Letters indicate statistically significant differences at $P < 0.05$ obtained by two-way ANOVA followed by the post-hoc Tukey's HSD. GxL value refers to the *P* value of the Genotype × Light interaction term in ANOVA.

phot1 Is Needed for Phototropism in LBL

The phyB-mediated phototropism in green seedlings is driven mainly by phot1. The *phot1* mutant is unable to bend in LRFR conditions, but *phot2* has the same phototropic response as wild-type seedlings (Goyal et al., 2016). Besides, phot1 is the major photoreceptor initiating phototropism toward relatively low blue intensities both in etiolated and light-grown seedlings (Christie et al., 2011). Therefore, we assessed whether phot1 is also involved in LBL and cry1-modulated phototropism. We compared the response of the *phot1cry1* double mutant with *cry1* and *phot1* single mutants (Fig. 3A). *phot1cry1* and *phot1* hypocotyls reoriented much less than wild type in persistent LBL (LBL/LBL), indicating that phot1 was needed for cry1-mediated phototropism enhancement. Moreover, NON-PHOTOTROPIC HYPOCOTYL3 (NPH3), which is essential for phototropism in etiolated and green seedlings (Motchoulski and Liscum, 1999; Goyal et al., 2016), was also required for the response in our conditions (Fig. 3B). One of the first steps in phot1 signaling is NPH3 de-phosphorylation, which has been recently implicated in modulating the phototropic response. Reduced NPH3 de-phosphorylation correlates with accelerated phototropism in seedlings treated for a few hours with light prior to phototropic stimulation (Sullivan et al., 2019). We therefore tested whether the LBL treatment that accelerates phototropism led to changes in NPH3 phosphorylation. NPH3 immunoblots did not reveal any differences among the tested light conditions, suggesting that the differences in hypocotyl curvature triggered by LBL were not a consequence of altered NPH3 phosphorylation status

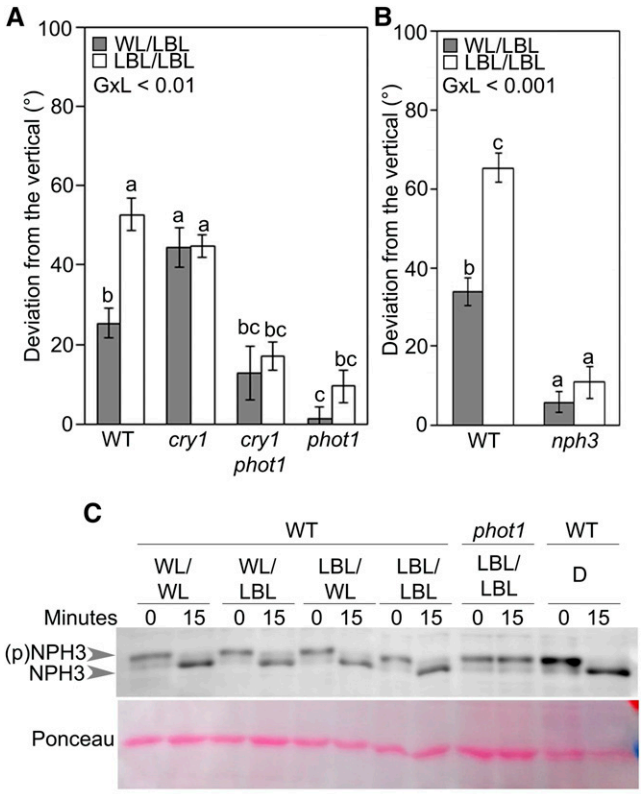

Figure 3. Phototropism in LBL requires phot1. Phototropism in wild-type (WT), *cry1*, *phot1*, and *cry1phot1* (A) and wild-type versus *nph3* seedlings (B). All measurements were conducted with 3-d-old seedlings 6 h after lateral blue light application. Bars represent means ($n \geq 25$) ± SE. Letters indicate statistically significant differences at $P < 0.05$ obtained by two-way ANOVA followed by the post-hoc Tukey's HSD. GxL value refers to the *P* value of the Genotype × Light interaction term in ANOVA. C, Detection of NPH3 phosphorylation state in 3-d-old dark-grown wild-type and *phot1-5* seedlings 0 and 15 min after dawn in the presence of lateral blue light (8 μmol $m^{-2}s^{-1}$). NPH3 was also detected in wild-type dark-grown seedlings (D) before (0) and after 15 min of lateral blue light (8 μmol $m^{-2}s^{-1}$). (p)NPH3 is the band corresponding to phosphorylated NPH3. Ponceau staining was used as loading control.

(Fig. 3B). We therefore conclude that LBL-enhanced phototropism requires phot1 and NPH3, but we have no evidence for a role of LBL-regulated NPH3 phosphorylation in this process.

PIF4 and PIF5 Modulate LBL-Dependent Phototropism Downstream of cry1

Cry act through PIFs to regulate hypocotyl elongation in response to temperature (Ma et al., 2016) and blue light (Pedmale et al., 2016). To understand if the cry1-PIFs module also operates during shade-controlled phototropism, we analyzed the phototropic bending of different combinations of *cry1* and *pif* mutants in three different light conditions with the same blue light gradient: WL/LBL, LBL/LBL, and WL/SCS. The *pif4pif5pif7* triple mutant had the same phototropic response as the wild type in WL/LBL but showed no phototropism enhancement in response to LBL pretreatment or SCS treatment the day of phototropism (Fig. 4A). Remarkably, the *pif4pif5pif7* triple mutant was epistatic over *cry1* in all tested conditions (Fig. 4A). Interestingly, *pif4pif5* double mutants were unresponsive to the LBL pretreatment, while they responded normally to the SCS treatment (Fig. 4A). Moreover, *pif4pif5* double mutants selectively suppressed the *cry1* phenotype in WL/LBL and LBL/LBL, but not WL/SCS conditions (Fig. 4A). To test the relevance of these findings in natural conditions, we analyzed the phototropic response outdoors in response to a real canopy (Fig. 4B). Seedlings were grown in the lab for 4 d before being placed on the south side of a grass canopy (southern hemisphere; Fig. 4B). Both *phyB* and *cry1* mutants reoriented more than wild-type seedlings, while *pif4pif5pif7* showed a weaker phototropic response (Fig. 4B). Interestingly, in this condition, *pif4pif5pif7*, but not *pif4pif5*, fully suppressed the *cry1* phenotype as observed in the lab in WL/SCS conditions (Fig. 4). Moreover, while *pif4pif5pif7* was fully epistatic over *cry1*, this triple mutant did not fully suppress the *phyB* phenotype (Fig. 4B). Taken together, our laboratory and outdoor experiments indicate that PIF4 and PIF5 are specifically required for the LBL response downstream of cry1. In contrast, the response to real canopy shade (LBL and LRFR) also requires PIF7 (Fig. 4; Goyal et al., 2016).

LBL Enhances PIF4 Protein Levels toward the End of the Day

Given the importance of PIF4 and PIF5 in regulating hypocotyl curvature (Fig. 4), we questioned whether the faster phototropic response observed under prolonged LBL (Fig. 2B) was accompanied with a faster accumulation of PIF4 and/or PIF5. We determined PIF4 (Fig. 5, A and B) and PIF5 (Fig. 5, A and C) protein levels using lines expressing the *PIF4/5-HA* transgene under the control of their native promoters (*PIF4p:PIF4-HA* in *pif4* and *PIF5p:PIF5-HA* in *pif5*) during the first 3 h of the phototropic response in LBL with or without LBL pretreatment. As reported previously (Bernardo-García et al., 2014; Galvão et al., 2019), levels of both PIF4 and PIF5 increased from ZT0 to ZT3, but we did not observe an effect of the LBL pretreatment on PIF protein levels (Fig. 5, B and C). Given that LBL-enhanced phototropism is most effective with an LBL pretreatment, we also determined whether this pretreatment altered PIF4 and PIF5 levels the day prior to the phototropic assay (Fig. 5, E and F). In WL, we observed diel regulation of PIF4 (Fig. 5E) and PIF5 (Fig. 5F), with a peak in the middle of the day (ZT8) and a decrease during the last hours of the day (ZT13–ZT17). LBL had a strong effect on PIF4 protein levels (Fig. 5, D and E). PIF4 levels remained high for much longer during the day and only returned to the same levels as in WL-treated samples at

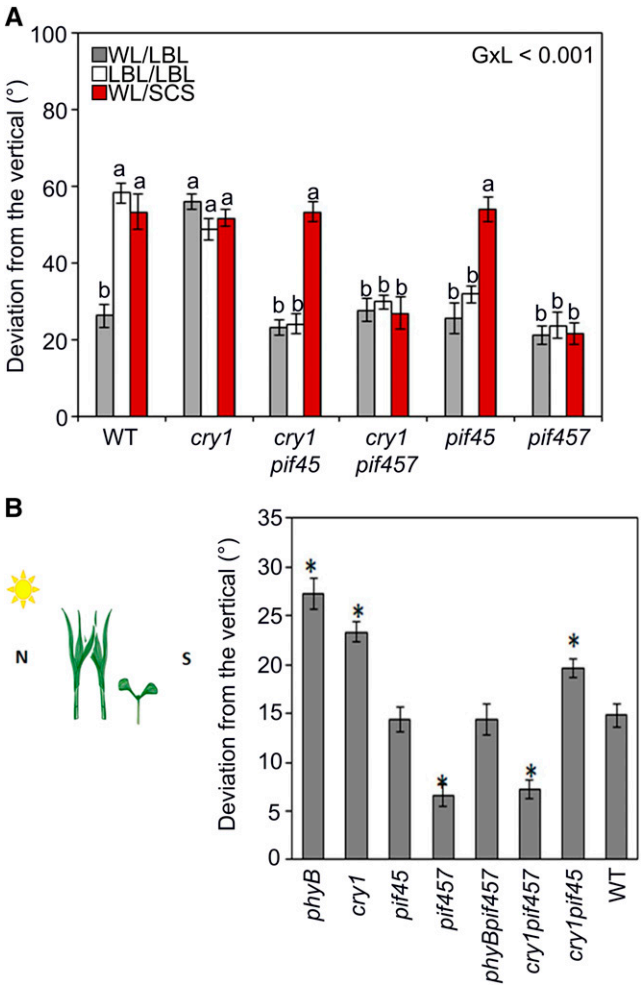

Figure 4. PIF4 and PIF5 act downstream of cry1 to control phototropism. A, Bars represent means ($n \geq 25$) ± SE values of wild type, *cry1*, *pif4pif5* (*pif45*), *pif4pif5pif7* (*pif457*), *cry1pif4pif5* (*cry1pif45*), and *cry1pif4pif5pif7* (*cry1pif457*) hypocotyl deviation from the vertical in the lab conditions with lighting regimes as described in Figure 1A. Letters indicate statistically significant groups at $P < 0.05$ obtained by two-way ANOVA followed by the post-hoc Tukey's HSD. GxL value refers to the *P* value of the Genotype × Light interaction term in ANOVA. B, Scheme of the outdoor experiment (left). The same seedling genotypes as in A plus *phyB* and *phyBpif4pif5pif7* (*phyBpif457*) were grown for 4 d in lab conditions and then moved to the south side of grass plants for phototropic assays. Hypocotyl bending was measured 5 h after phototropic stimulation. Bars (right) represent mean values ± SE of three independent experiments. Asterisks indicate the statistical significance by Student's *t* test of mutant bending with respect to wild type (*$P < 0.05$).

ZT19 (Fig. 5E). LBL had a more modest effect on PIF5 protein levels, which declined slightly slower in LBL than in WL conditions (Fig. 5F). As a control, we probed the membrane with CRY1 and CRY2 antibodies. As reported previously (Shalitin et al., 2002), LBL led to higher levels of CRY2 protein but not CRY1 (Fig. 5, E and F). We conclude that LBL has a strong effect on PIF4 protein levels, particularly toward the end of the day.

cry1 Modulates the Abundance of PIF4

To determine how LBL regulates PIF protein abundance, we first determined the effect of this light treatment on *PIF* transcript abundance using reverse transcription quantitative PCR. *PIF4* (Fig. 6A), but not *PIF5* (Supplemental Fig. S3), transcript levels increased in LBL, as described previously (Pedmale et al., 2016). However, the LBL treatment did not alter the diel expression profile of *PIF4* and *PIF5* (Fig. 6A; Supplemental Fig. S3). *PIF4* levels were higher in WL-grown *cry1* mutants than in the wild type with *cry1* mutants expressing *PIF4* at a level similar to LBL-grown wild-type seedlings (Fig. 6A). The negative effect of cry1 on PIF4 abundance was also observed by immunoblotting using a PIF4 antibody (Fig. 6B). This effect on PIF4 protein abundance was confirmed and quantified comparing PIF4-HA in the wild type versus *cry* mutant background. This experiment showed that PIF4-HA levels were higher in *cry1* and *cry1cry2* particularly in WL conditions (Supplemental Fig. S4A). Moreover, PIF4-HA levels were not altered in etiolated *cry1* mutants (Supplemental Fig. S4B), indicating that cry1 regulates PIF4 levels in response to light. The *PIF4p:PIF4-HA* line expressed higher levels of PIF4 than the wild type (Supplemental Fig. S4C). This provided us with an opportunity to test whether higher PIF4 levels were sufficient to promote phototropism. Interestingly, the phototropic response of *cry1*, *PIF4p:PIF4-HA* and *PIF4p:PIF4-HAcry1* was very similar, with enhanced bending compared to the wild type in WL/LBL conditions and no additive effects observed in *PIF4p:PIF4-HA cry1* (Supplemental Fig. S4D). This indicates that higher PIF4 levels, as observed in *cry1* or *PIF4p:PIF4-HA*, promoted phototropism in WL/LBL, but further increasing PIF4 levels, as in *PIF4p:PIF4-HAcry1*, did not further enhance the bending response. Overexpression of PIF5 under the control of 35S promoter also enhanced phototropism in WL/LBL conditions (Supplemental Fig. S4E). Taken together these data underline the importance of PIF4 and PIF5 levels in the control of cry1-modulated phototropism.

Our experiments indicate that high cry1 activity and low PIF4 levels limit phototropism in high light (WL) conditions. This model predicts that a mutant with high cry activity will have reduced PIF4 levels and be less responsive to blue light gradients. We tested this using the *bic1bic2* (*b1b2*) double mutant, which has higher cry activity (Wang et al., 2016). *b1b2* had the same phototropic response than the wild type in WL/LBL conditions. However, in persistent LBL, which strongly promotes phototropism in the wild type, *b1b2* showed a reduced phototropic response and reduced levels of PIF4 (Fig. 6, C and D). Taken together our data indicate that cry1 controls phototropism at least in part by controlling PIF4 levels.

Phototropism in LBL Requires Auxin Transport, but Also Biosynthesis and Signaling

Asymmetrical hypocotyl growth is ensured by differential auxin distribution, which is mediated by several

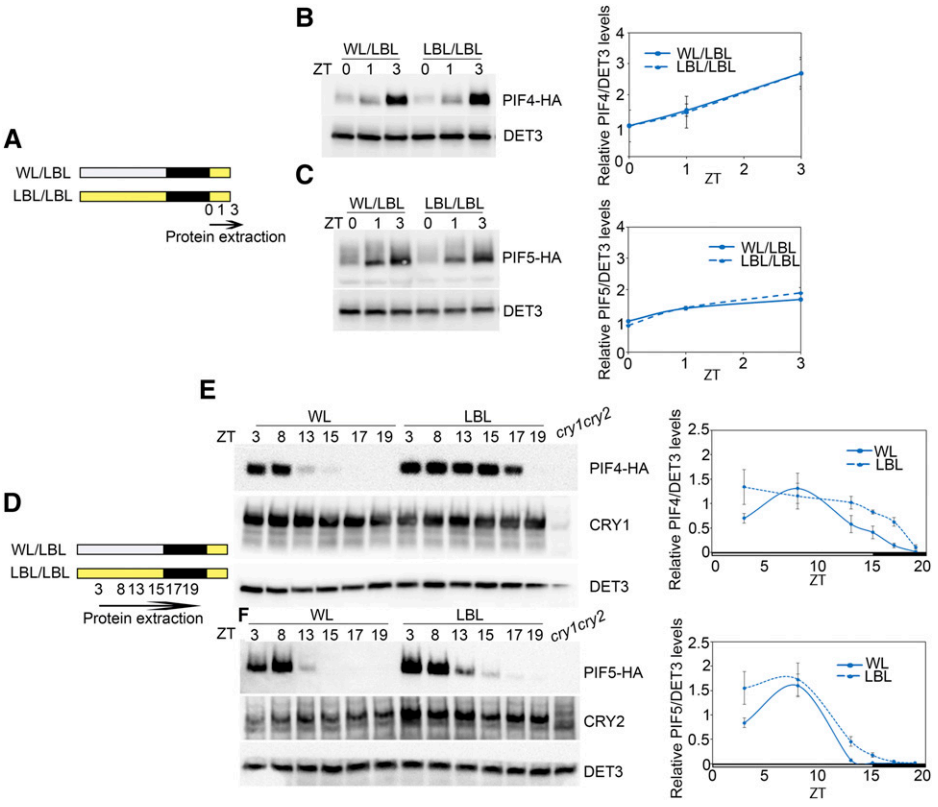

Figure 5. LBL leads to PIF4 and PIF5 accumulation. A and D, Schematic representations of the last 2 d of the experiment. White boxes represent WL treatment and yellow boxes LBL. Black boxes represent the night. B and C, Immunoblot for PIF4-HA and PIF5-HA detected by HA antibody in *PIF4p:-PIF4-HA* (B) and *PIF5p:PIF5-HA* (C) seedlings pretreated or not with LBL the previous day and harvested at ZT0, ZT1, and ZT3. E and F, Detection of PIF4-HA (E) and PIF5-HA (F) at ZT3, ZT8, ZT13, ZT15, ZT17, and ZT19 during LBL treatment. In the immunoblot quantifications on the right, PIF4-HA and PIF5-HA levels are normalized for the loading control DET3 and are relative to WL ZT0 (B and C) or to WL ZT3 (E and F) samples fixed to 1. Values are the average of three independent experiments ± SE. DET3 was used as loading control. CRY1 (E) and CRY2 (F) were detected by antibodies against the endogenous proteins.

classes of auxin transporters, including PINs (Liscum et al., 2014). Consistent with these findings, the *pin3-pin4pin7* triple mutant responded less than wild type in all conditions analyzed (Fig. 7A). The analysis of an epidermis-specific variant of the ratiometric auxin reporter quantitative DII-Venus (Galvan-Ampudia et al., 2019) 1 h after phototropic stimulation (Fig. 7B) revealed the presence of an auxin signaling gradient across the hypocotyl both in WL/LBL and LBL/LBL (Fig. 7C). Interestingly, in persistent LBL conditions, the gradient was steeper paralleling with the faster phototropic response (Figs. 2 and 7C). Moreover, before phototropic stimulation, the hypocotyl of seedlings pretreated for 24 h in LBL showed a lower quantitative DII-Venus value compared to seedlings kept in WL (Fig. 7D). A low quantitative DII-Venus value can be caused by higher auxin levels or higher activity of the TIR1/AFB auxin receptors, and both of these aspects may explain the faster phototropic response in persistent LBL. In response to LRFR, PIF proteins promote new auxin biosynthesis trough transcriptional activation of *YUC* genes (Hornitschek et al., 2012; Li et al., 2012). *yuc2yuc5yuc8yuc9*, as well as *taa1/sav3*, were less responsive to long LBL treatments (Fig. 7E), suggesting that in persistent LBL, new auxin biosynthesis is also needed for a full phototropic response. Moreover, the reduced phototropic response in persistent LBL of *msg2* (Fig. 7F) indicates involvement of auxin-mediated degradation of auxin/indole-3-acetic acid (Aux/IAA) proteins. The mutant for the auxin receptor TIR1 also showed a reduced phototropic response (Fig. 7G). However, the *tir1* phenotype was not specific to a particular light treatment (the statistical interaction Genotype × Light was not significant), suggesting that TIR1 is not selectively required for phototropism in LBL. We propose that LBL enhancement of phototropism results from a steeper auxin-signaling gradient across the hypocotyl, which may result from a coordinate action on auxin synthesis, transport, and/or signaling.

DISCUSSION

Canopy Shade Promotes Phototropism with a Strong Contribution of LBL

A positive correlation exists between dense vegetation and phototropism (Ballaré et al., 1992), and the inactivation of phyB by LRFR enhances phot1-mediated hypocotyl reorientation toward directional blue light (Goyal et al., 2016). These experiments also show that phototropism is strongly enhanced at a very low R to FR ratio (0.2) that is typical of canopy shade and not reached prior to actual shading in neighbor proximity conditions (Ballaré et al., 1990; Fiorucci and Fankhauser, 2017). We therefore investigated the effect of different features of canopy shade by testing the effects of either LRFR, LBL, and the combination of both (SCS), which mimics true shade in lab conditions (de Wit et al., 2016). These experiments showed that only SCS leads to rapid promotion of phototropism (Fig. 1B),

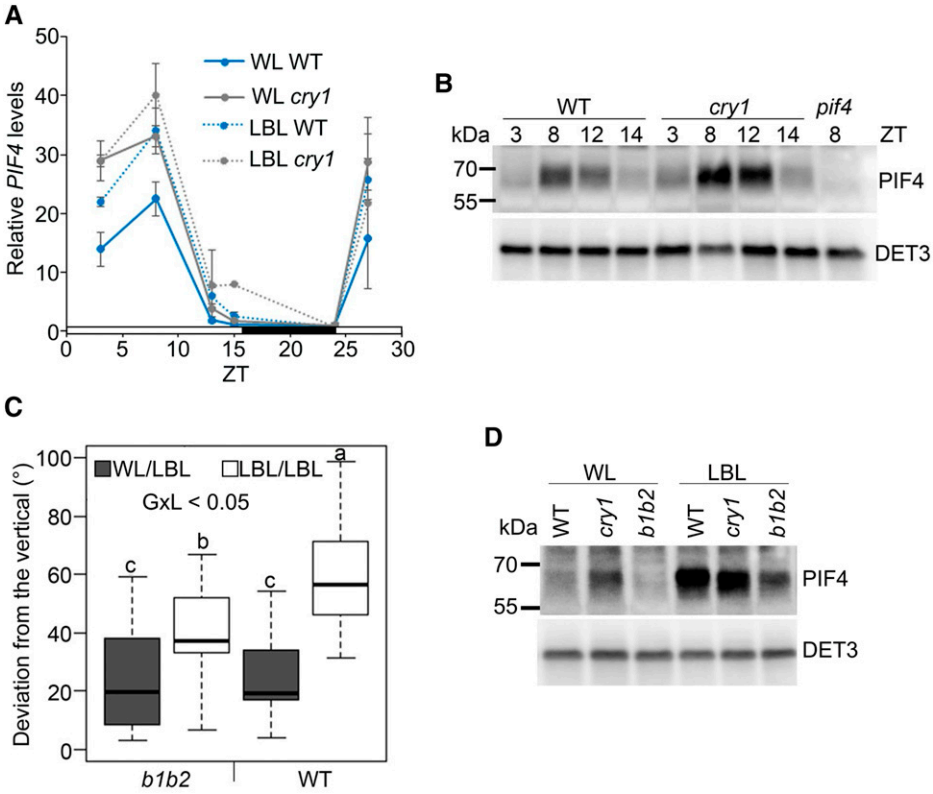

Figure 6. cry1 is involved in the regulation of PIF4 levels. A, Reverse transcription quantitative PCR analysis for *PIF4* in 4-d-old seedlings kept in WL or moved to LBL at ZT0. RNA was extracted at ZT3, ZT8, ZT13, ZT15, ZT24, and ZT27 from wild-type (WT) and *cry1* seedlings. Values represent the average of two independent experiments ± SE. B, Immunoblot for protein extracted from wild-type and *cry1* 4-d-old seedlings grown in WL at the indicated hours during the day. *pif4* mutant sample at ZT8 was used to check the specificity of the PIF4 band. DET3 was used as loading control. C, Phototropic assay of wild-type and *bic1bic2* (*b1b2*) seedlings. Measurements were conducted in 3-d-old seedlings 6 h after lateral blue light application. Letters indicate statistically significant differences at $P < 0.05$ obtained by two-way ANOVA followed by the post-hoc Tukey's HSD ($n \geq 25$). GxL value refers to the *P* value of the Genotype × Light interaction term in ANOVA. D, Immunoblot for endogenous PIF4 levels in samples collected at ZT13 kept in WL or moved to LBL at ZT0 in. DET3 was used as loading control.

indicating a synergistic effect of LBL and LRFR on phototropism. The apparent contradiction between these results and our previous work can be explained by the very low light environment in which we performed our earlier experiments (plates were positioned in black boxes with only an opening on one side in Goyal et al., 2016). We therefore conclude that phototropism enhancement is triggered by actual vegetational shade (LRFR and LBL) rather than by neighbor proximity alone (LRFR without LBL).

Our experiments revealed that LBL strongly contributes to phototropic enhancement. For LBL to be effective on its own, it is required for several hours the day prior and during phototropic stimulation (Fig. 1B; Supplemental Fig. S1). This might be due to the slower effect of LBL, compared to LRFR, in promoting hypocotyl elongation (Pedmale et al., 2016). However, phototropic enhancement does not simply depend on hypocotyl elongation, given that prolonged LRFR, which is highly effective in promoting hypocotyl elongation, does not promote phototropism (Supplemental Fig. S1). The fact that LBL alone when applied from the day prior to phototropic stimulation was sufficient to promote phototropism allowed us to specifically study the role of this component of canopy shade in phototropism enhancement. Altering blue light from above the plant to generate LBL also modifies the horizontal blue light gradient in our experimental setup (Fig. 1A). However, several experiments allowed us to demonstrate that phototropism enhancement in LBL is not simply a consequence of a modified light gradient (Fig. 1; Supplemental Fig. S2). We conclude that ambient LBL is an important feature of canopy shade enhancing phototropism.

cry1 Has a Negative Effect on Hypocotyl Reorientation of Green Seedlings

Our experiments show that in de-etiolated seedlings cry1 inhibits phototropism in favorable (WL) light conditions (Fig. 2; Supplemental Fig. S2). In the conditions we tested, phot1 is the primary photoreceptor

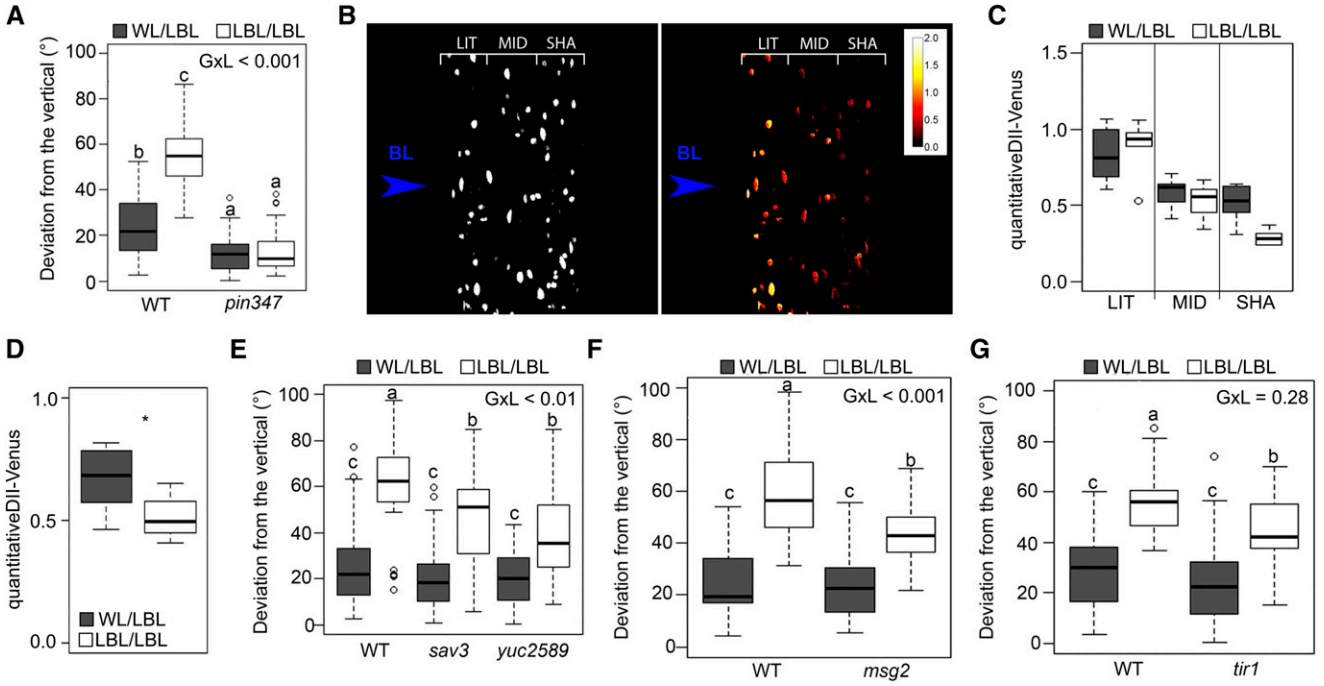

Figure 7. Auxin transport, biosynthesis, and signaling have a role in LBL-enhanced phototropism. A, Phototropic assay of *pin3pin4pin7* (*pin347*) mutant. B, C, and D, Quantification of auxin signaling using the fluorescent ratiometric auxin signaling report pPDF1::DII-n7-Venus-2A-mTurquoise-sv40. Seedlings were grown as in Figure 1, with LBL or WL conditions the day before the phototropic assay, and transferred to LBL during the phototropic assay. Confocal images were taken from the epidermal in the elongation zone of the hypocotyl the last day of the experiment. B, Representative confocal images showing the nuclei expressing the sensor according to the mTurquoise fluorescence (left) and the quantitative DII-Venus values calculated as the ratio between Venus and mTurquoise fluorescence (right) after the phototropic assay in LBL/LBL. The blue arrow represents the direction of the phototropic stimulus. The color code represents the quantitative DII-Venus value in cells facing the light (LIT), in the middle of the hypocotyl (MID), or in the side opposite the light, shaded side (SHA). Lower quantitative DII-Venus levels indicate higher auxin signaling. C, Quantitative DII-Venus quantification 1 to 2 h after the phototropic assay. D, Quantification of quantitative DII-Venus before the phototropic assay in the MID region. Asterisks indicate statistical significance by Student's *t* test (*$P < 0.05$). Phototropic assay of mutants for auxin biosynthesis (*sav3* and *yuc2yuc5yuc8yuc9* [*yuc2589*], E) and signaling (*msg2* [F] and *tir1* [G]). The deviation from the vertical was measured 6 h after lateral blue light application. Letters indicate statistically significant differences at $P < 0.05$ obtained by two-way ANOVA followed by the post-hoc Tukey's HSD ($n \geq 25$). GxL value refers to the *P* value of the Genotype × Light interaction term in ANOVA.

controlling hypocotyl reorientation, and the enhanced response of *cry1* mutants depends on phot1 (Fig. 3). Light promotes *PHOT2* and represses *PHOT1* expression (Łabuz et al., 2012). Phot1 protein levels also decrease after blue light exposure (Kong et al., 2006; Kozuka et al., 2011; Łabuz et al., 2012), indicating that light activates phototropins and regulates their expression. Hence, the LBL-enhanced phototropism reported here might be a consequence of changes in *PHOT1* and/or *PHOT2* expression. However, the analysis of LBL-regulated gene expression performed in conditions very similar to the ones used here (Pedmale et al., 2016) revealed no obvious effect on *PHOT1* and *PHOT2* expression. Given that one of the first events occurring after phot1 activation is NPH3 dephosphorylation and NPH3 phosphorylation affects phototropism in seedlings treated with a few hours of light to initiate de-etiolation (Sullivan et al., 2019), we investigated NPH3 regulation in our conditions. NPH3 was essential for phototropism, but we did not detect an effect of LBL on NPH3 phosphorylation (inferred from mobility shifts on SDS-PAGE gels; Fig. 3). Further analysis will be necessary to clarify whether the reduction of blue light in a canopy shade situation can directly affect other steps of the phot1 signaling pathway.

Cry1-mediated phototropic suppression depends on PIF transcription factors. Under real canopy shade experiments performed outdoors, the *pif4pif5pif7* triple mutant was fully epistatic over *cry1*, while the *pif4pif5* double mutant partially suppressed *cry1* (Fig. 4B). Similarly, in SCS conditions, we also found that *cry1* was only suppressed by the *pif4pif5pif7* triple mutant and not the *pif4pif5* double mutant (Fig. 4A). However, when focusing on LBL, the *pif4pif5* double mutant was sufficient to suppress *cry1* (Fig. 4A), consistent with previous studies, which identified PIF4 and PIF5 as the major PIFs acting downstream of cry1 in controlling shade responses (Keller et al., 2011; Pedmale et al., 2016).

cry1 Inhibits *PIF4* Expression to Control Phototropism

The importance of PIF4 and PIF5 in controlling LBL-induced phototropism downstream of cry1 prompted us to analyze PIF4/PIF5 regulation by light and cry1. LBL treatment led to elevated PIF4-HA and to a lesser extent PIF5-HA toward the end of the day (Fig. 5). These data were confirmed for PIF4 using an antibody recognizing the endogenous protein (Fig. 6). PIF4 seems to have a predominant role in regulating hypocotyl elongation in LBL; in fact, *pif4* elongates similarly to the *pif4,5* double mutant but less than *pif5*, and *pif4* alone abolishes the *cry1* elongation phenotype (Pedmale et al., 2016). Our data showed that cry1 regulates PIF4 levels, as shown by the analysis of PIF4 levels in *cry1* and *bic1bic2* mutants. The *cry1* mutant has higher PIF4 levels than the wild type in WL conditions, while the *bic1bic2* double mutant, with higher cry activity (Wang et al., 2016, 2017), has lower PIF4 levels in LBL (Fig. 6D). This observation correlates with a reduced phototropic response of the *bic1bic2* double mutant (Fig. 6C). The effect of LBL and *cry1* on PIF4 levels could, at least in part, be due to transcriptional regulation given that *PIF4* transcript levels were higher in LBL than in WL conditions (Fig. 6A). Moreover, LBL-regulated *PIF4* levels were essentially absent in *cry1* mutants, which always expressed higher *PIF4* levels than WL-treated wild type (Fig. 6A). These data are consistent with previous studies showing cry1-mediated transcriptional regulation of *PIF4* in monochromatic blue light (Ma et al., 2016; He et al., 2019). The control of PIF4 levels by cry1 is light regulated, given that we observed no effects of *cry1* on PIF4 levels in etiolated seedlings (Supplemental Fig. S4). Interestingly, a *PIF4p:PIF4-HA* line, which expresses higher PIF4 levels than the wild type, has a very similar phototropic phenotype to *cry1* without a further enhancement of the phototropic response in the *cry1 PIF4p:PIF4-HA* line (Supplemental Fig. S4). This suggests that high levels of PIF4 alone are sufficient to promote phototropism and that a major level of cry1 regulation is the transcriptional control of PIF4 accumulation. These observations are in agreement with previous data showing that when expressed from a constitutive promoter, PIF4 protein levels are unchanged in the *cry1* mutant (Ma et al., 2016). The precise mechanism underlying cry1-mediated enhancement of *PIF4* expression remains unknown. However, it is noteworthy that cry2 modulates gene expression in a blue-light-regulated fashion when expressed in a heterologous system (Yang et al., 2018). Given that we also observed a modest effect of LBL on PIF5-HA protein levels, we do not rule out additional levels of PIF4 and PIF5 regulation by cry1, such as posttranscriptional regulation or inhibition of PIF4 and PIF5 activity (Ma et al., 2016; Pedmale et al., 2016). Yet, the striking association between cry1-mediated PIF4 accumulation and LBL-modulated phototropism highlights the importance of cry1-regulated PIF4 abundance at the transcriptional level.

The Importance of Auxin for LBL-Mediated Phototropic Enhancement

Several reports have demonstrated impaired hypocotyl elongation responses to LBL in mutants defective in auxin transport and auxin biosynthesis (Pierik et al., 2009; Keuskamp et al., 2011; de Wit et al., 2016). Deficient enhancement of the phototropic response by LBL in the *sav3*, *yuc2yuc5yuc8yuc9*, *pin3pin4pin7*, and *msg2* mutants (Fig. 7) indicates that this process requires normal auxin synthesis, transport, and signaling. A priori, the phenotype of these mutants might simply indicate that normal auxin synthesis, transport, and signaling are a condition for the LBL effects or that the auxin system carries LBL information. In this regard, PIFs regulate auxin signaling in response to light stimuli at multiple levels, including biosynthesis, transport, perception, and signaling (Oh et al., 2014; Kohnen et al., 2016; Iglesias et al., 2018; Pucciariello et al., 2018), and therefore, LBL-mediated phototropic enhancement may affect more than one of these levels of regulation. For instance, shade cues (LBL and/or LRFR) promote the expression of several *PIN*s, including *PIN3* and *PIN7* (Keuskamp et al., 2011; Kohnen et al., 2016). Moreover, cry1 and the PIFs regulate *PIN* expression in an antagonistic way, with higher expression in *cry1* mutants and reduced expression in *pif* mutants (Hornitschek et al., 2012; Li et al., 2012; He et al., 2019). Given that PIF4 and PIF5 directly bind to the promoter of *PIN3* (Hornitschek et al., 2012), it is possible that the cry1-mediated regulation of PIF4 abundance modulates the phototropic response via PIF-controlled *PIN* expression. At least under LRFR, PIFs also promote the expression of several *YUC* genes to enhance phototropism (Goyal et al., 2016). Moreover, prolonged shade treatment induces remodeling of auxin signaling, which includes changes in *IAA19*, *IAA29*, and *IAA17* expression. These changes ensure hypocotyl elongation also during the second day of treatment, when an increase of auxin levels is not anymore detected (Pucciariello et al., 2018). We used quantitative DII-Venus to investigate whether the auxin system carries the LBL information. Our data indicate that the latter is actually the case because an LBL pretreatment leads to higher auxin levels and/or sensitivity in the hypocotyl (Fig. 7C) and a steeper gradient of auxin levels and/or sensitivity upon phototropic stimulation (Fig. 7B), which correlates with enhanced phototropism (Fig. 1). Considering the long-term effect of LBL on phototropism, it is possible that the accumulation of PIF4 and PIF5 proteins the first day of LBL leads to a remodeling of auxin availability (biosynthesis and transport) and signaling (AUX/IAA), which would make seedlings more responsive to phototropic stimuli the day after. However, LBL has to be maintained also during the day of phototropism to have a robust and persistent curvature (Fig. 2), indicating that the inhibitory effect of blue light on cell elongation can suppress this effect.

In natural canopies, LBL never occurs alone but is always associated with LRFR. Our observations point

out LBL as the limiting step to enhance hypocotyl reorientation in canopy shade, suggesting that LBL carries extra information about the environment. Individually, both LRFR and LBL act on the PIF and auxin pathways, and they have a similar effect on the promotion of hypocotyl elongation (Supplemental Fig. S1B) but different effects on phototropism (Fig. 1; Supplemental Fig. S1A). However, when LRFR and LBL are combined (SCS condition in our study; Fig. 1) they have a synergistic effect on hypocotyl reorientation. This aspect raises interesting questions about the differences between LRFR and LBL response. Does LBL boost the response activated by LRFR acting on the same pathways? Or, does LBL affect alternative pathways? Do LBL and LRFR perception occur in the same tissues and/or organs? Further analysis will be necessary to clarify how hypocotyl elongation and reorientation are coordinated in different light environments.

We conclude that phototropic enhancement by canopy shade involves changes in activity of at least three photoreceptors: phot1, cry1, and phyB (Figs. 2 and 3; Goyal et al., 2016). In shade, the reduced activity of cry1 and phyB permits enhanced PIF abundance, leading to a modification of auxin signaling status in the hypocotyl to promote phototropism when phot1 perceives the blue-light gradient (Fig. 7; Goyal et al., 2016).

MATERIALS AND METHODS

Plant Material

The following Arabidopsis (*Arabidopsis thaliana*; Col-0 ecotype) mutants were previously characterized: *cry1-304* (Mockler et al., 1999); *phot1-5* (Huala et al., 1997); *nph3-6* (Motchoulski and Liscum, 1999); *cry2-1* (Guo et al., 1998); *cry1-304cry2-1*, *cry1-304pif4-101pif5-3*, *cry1-340pif4-301pif5-3pif7-1*, and *cry1-phyB* (Fiorucci et al., 2020); *phyB-9*, *phyB-9pif4-101pif5-3*, and *phyB-9pif4-101pif5-3pif7-1* (Goyal et al., 2016); *pif4-101pif5-3* and *OXPIF5* (Lorrain et al., 2008); *pif4-101pif5-3pif7-1* (de Wit et al., 2015); *PIF4p:PIF4-HApif4-301* (Galvão et al., 2019); *PIF5p:PIF5-HApif5-3* (de Wit et al., 2016); *bic1bic2* (Wang et al., 2016); *sav3/taa1* (Tao et al., 2008); *yuc2yuc5yuc8yuc9* (Kohnen et al., 2016); *tir1-1* (Ruegger et al., 1998); *msg2* (Tatematsu et al., 2004); and *pin347* (Willige et al., 2013). *pPIF4:PIF4-HApif4-101cry1-304*, *pPIF4:PIF4-HApif4-101cry1-304cry2-1*, and *cry1-304phot1-5* were obtained by crosses, and the primers used for genotyping are listed in Supplemental Table 1. The epidermis-specific promoter protodermal factor1 (PDF1; Abe et al., 2001) driving the expression of a DII-VENUS-N7-2A-TagBFP-sv40 (quantitative DII-Venus; Galvan-Ampudia et al., 2019) was assembled by Gateway technology (Invitrogen) and transformed in Arabidopsis (Col-0).

Growth and Light Conditions

Seeds were surface-sterilized in 70% (v/v) ethanol and 0.05% (v/v) Triton X-100 for 10 min and washed once with 100% (v/v) ethanol. After sterilization, seeds were placed on plates (10 cm × 10cm) containing 40 mL of one-half strength Murashige and Skoog medium, 0.8% (w/v) phytoagar (Agar-Agar, plant; Roth), and MES. After 2 d of stratification (4°C and dark), seedlings grew vertically inside customized black boxes. To avoid variability due to the light gradient toward the bottom of the black boxes during seedlings growth, only 32 seeds were sowed in the upper part of the plate arranged in four rows. Seedlings were grown in long day (16 h day/8 h night, 21°C/19°C) in a plant growth incubator equipped with fluorescent bulbs in the presence of 95 μmol $m^{-2}s^{-1}$ of photosynthetic photon flux density, measured by a white diffuser filter combined with a PAR filter (Radiometer model IL1400A, https://www.intl-lighttech.com/product-group/light-measurement-optical-filters). The single layer of yellow filter (010 medium yellow, LEE Filters) used to cover up the seedlings decreased blue light from 7 μmol $m^{-2}s^{-1}$ (WL) to 0.7 μmol $m^{-2}s^{-1}$ (LBL), and it was measured by a white diffuser filter combined with a blue filter (400–500 nm). The R (640–700 nm)/FR (700–760 nm) ratio was measured using a spectrometer (Ocean Optics USB2000+). The light spectra are shown in Supplemental Figure S5. In WL and LBL, the R/FR ratio was 1.25, and in LRFR and SCS it was 0.3, obtained by adding FR LED to WL lamps. The LEE filter no. 298 0.15 ND used in Supplemental Figure S1 reduced PAR similarly to the yellow filter used for LBL treatment: transmission through 0.15-ND filter was 69.3%, the transmission of PAR through the yellow filter was about 76.6%. Blue light measured in the presence of 0.15-ND filter was 4.9μmol $m^{-2}s^{-1}$.

Phototropism

Phototropic stimulation by the application of lateral blue light was always started after the lights turned on, between ZT0 and ZT0.5, by removing one side of the black boxes and supplying unilateral blue light by LEDs. The LED source was placed 60 cm distant from the black boxes to increase the horizontal blue light to 8 μmol $m^{-2}s^{-1}$. In addition, the blue light differential between the illuminated side and the top (i.e. the light coming from above) was calculated. Because the yellow filter in the LBL condition affected this differential, its potential physiological impact was evaluated. The log10 of the side-to-top differential of blue light was 0.0 (log10 (side blue light (8 μmol $m^{-2}s^{-1}$) − top blue light (7 μmol $m^{-2}s^{-1}$)) = 0) under WL (high blue light from above) and 0.9 (log10 (side blue light (8 μmol $m^{-2}s^{-1}$) − top blue light (0.7 μmol $m^{-2}s^{-1}$)) = 0.9) under LBL. Only in the WL condition presented in Supplemental Fig. S2B, the side blue light was increased up to 24 μmol $m^{-2}s^{-1}$ to obtain a log10 of the differential between the illuminated side and the top similar to that observed under LBL conditions (log10 (side blue light (24 μmol $m^{-2}s^{-1}$) − top blue light (12 μmol $m^{-2}s^{-1}$) = 1.0).

Measurement of the Phototropic Response

Pictures taken before and after phototropism were analyzed with a MATLAB script developed to obtain bending angle and hypocotyl length values. The bending angle was calculated as the deviation from the vertical of the upper part of the hypocotyl (75%−95% of the hypocotyl length). Since both *phot1cry1* and *phot1* are impaired in their gravitropic response, the seedlings were straightened before phototropism and the bending angle was calculated between T0 and T6 h of treatment. For the creation of box plots and to compute the one- or two-way ANOVA (aov) and Tukey's honest significance differences (HSD.test), the [agricolae package] of the R software was used. Similar results were obtained in three independent experiments.

RNA Extraction and Gene Expression Analysis

First, 20 to 25 seedlings grown on horizontal petri dishes were frozen in liquid nitrogen, and total RNA was extracted with the RNeasy mini kit (Qiagen). cDNA was prepared from 500 ng of RNA using superscript reverse transcriptase (Invitrogen) and random primers (0.25 μg μL^{-1}). A 1:20 cDNA dilution was mixed with Power SYBR green PCR master mix (Applied Biosystems) and primer mix at the final concentration of 0.3 μM. Three technical replicates were loaded on a 384 PCR plate using TECAN liquid handling system and run on a QuantStudio 6 flex system (Thermo Fisher Scientific). Relative gene expression was calculated as $(Eff_{(TARGET)})^{\Delta CtTARGET}/(Eff_{(HK)})^{\Delta CtHK}$. In the calculation, the average of the Ct values derived from two housekeeping genes (*UBC* and *YSL8*) was used to normalize the expression of the target gene. The primer sequences are listed in Supplemental Table S1.

Protein Extraction and Immunoblot Analysis

Total proteins were extracted in liquid nitrogen from 20 to 25 seedlings grown horizontally on petri dishes. For the detection of HA (1:2000, coupled with horseradish peroxidase [HRP], Roche, catalog no. 12013819001), CRY1 (1:4000, anti-rabbit secondary antibody; Lin et al., 1996), CRY2 (1:3000, anti-rabbit secondary antibody; Lin et al., 1998), and NPH3 (1:3000, anti-rabbit secondary antibody; Motchoulski and Liscum, 1999) proteins were extracted in 90 μL of protein extraction buffer (125 mM Tris, pH 6.8, 4% [w/v] SDS, 20% [v/v] glycerol, 0.02% [w/v] bromophenol blue, 10% [v/v] β-mercaptoethanol). For the detection of PIF4 N3 (1:3000, Abiocode R2534-4), modified protein extraction buffer (100 mM Tris-HCl, pH 6.8, 5% [w/v] SDS, 20% [v/v] glycerol, 80 μM MG132, 20 mM DTT, 1× protease inhibitor cocktail [P9599; Sigma-Aldrich], 1 mM bromophenolblue) was used. Samples were heated for 5 min at 95°C and

centrifuged for 1 min at 15,000*g* at room temperature before separation on 4% to 20% MiniProtean TGX gels (Bio-Rad, catalog no. 4561096), except for the NPH3 immunoblot, for which 8% acrylamide SDS-PAGE gels were used. Ten microliters of each sample was loaded. All samples were transferred to a nitrocellulose membrane with the Trans-Blot Turbo RTA transfer kit (Bio-Rad, catalog no. 170-4270). Next, 5% (w/v) milk dissolved in phosphate-buffered saline with 0.1% (v/v) Tween 20 was used for blocking for 1 h at room temperature and antibody dilutions, except for the anti-PIF4 N3 antibody, for which blocking was conducted overnight at 4°C to reduce background. HRP-conjugated anti-rabbit immunoglobulin (1:5000; Promega, catalog no. W4011) was used as a secondary antibody for CRY1, CRY2, DET3, and PIF4 N3. DET3 (1:20,000, anti-rabbit secondary antibody; Schumacher et al., 1999) was used as a loading control. The chemiluminescent signal was detected with Immobilon western chemiluminescent HRP substrate (Millipore) on an ImageQuant LAS 4000 mini (GE Healthcare). The HA/DET3 signals were quantified using ImageJ software.

DII-Venus Detection and Quantification

The genotype used was pPDF1::DII-n7-Venus-2A-mTurquoise-sv40 t35 in the Col-0 background. pPDF1 drives the expression of the sensor in the epidermis. This line expresses the DII degron fused to the yellow fluorescent protein Venus, which is degraded in response to auxin perception by its receptor under the same promoter as the blue fluorescent protein mTurquoise, which is not degraded in response to auxin. This allows quantification of auxin signaling levels as the ratio of yellow to blue fluorescence (qDII-Venus), independently of the expression levels of the selected promoter in the selected cells. Seedlings were grown as for the phototropic experiment. On the fourth day, half of the plates were shifted to LBL and half of them were kept in WL. On the fifth day, all plates were covered with a yellow filter before the start of the day, and 1 h after the start of the day, one side of the black box was opened to perform the phototropism assay. Confocal images were taken between ZT23.5 and ZT0.5 and between 1 and 2 h after starting the phototropic assay. All pictures were taken in the epidermis in the elongation zone using an LSM710 confocal microscope (Zeiss) equipped with an EC Plan-Neofluar 20×/0.50 objective. For Venus, an Argon laser (514 nm) was used and detected between 519 and 559 nm. For mTurquoise, a 405-nm diode laser was used and detected between 430 and 470 nm. Image analysis was performed in ImageJ. A threshold was applied to the mTurquoise channel to segment the nuclei. Mean intensity was measured inside each nucleus in the mTurquoise and the Venus channels, and qDII-Venus was calculated as the ratio between these two values for each nucleus. Each data point represents the average of three to four seedlings coming from one plate, and at least five nuclei were measured in each seedling. In Figure 7, B and C, nuclei were grouped according to their position in the hypocotyl with respect to the unilateral light stimulus.

Accession Numbers

The Arabidopsis Genome Initiative numbers for the genes mentioned in this article are as follows: AT4G08920 (*CRY1*), AT1G04400 (*CRY2*), AT3G45780 (*PHOT1*), AT5G64330 (*NPH3*), AT2G43010 (*PIF4*), AT3G59060 (*PIF5*), AT5G61270 (*PIF7*), AT2G18790 (*PHYB*), AT3G52740 (*BIC1*), AT3G44450 (*BIC2*), AT1G70940 (*PIN3*), AT2G01420 (*PIN4*), AT1G23080 (*PIN7*), AT1G70560 (*SAV3*), AT4G13260 (*YUC2*), AT5G43890 (*YUC5*), AT4G28720 (*YUC8*), AT1G04180 (*YUC9*), AT3G15540 (*IAA19*), and AT3G62980 (*TIR1*).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Prolonged LRFR treatment promotes elongation, but not phototropism.

Supplemental Figure S2. The reduction of blue light in the environment is a specific signal-enhancing phototropism.

Supplemental Figure S3. *PIF5* expression is not affected by LBL.

Supplemental Figure S4. High levels of PIF4 enhance phototropism.

Supplemental Figure S5. Light spectra of the conditions used in this study.

Supplemental Table S1. Primers used in this study.

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