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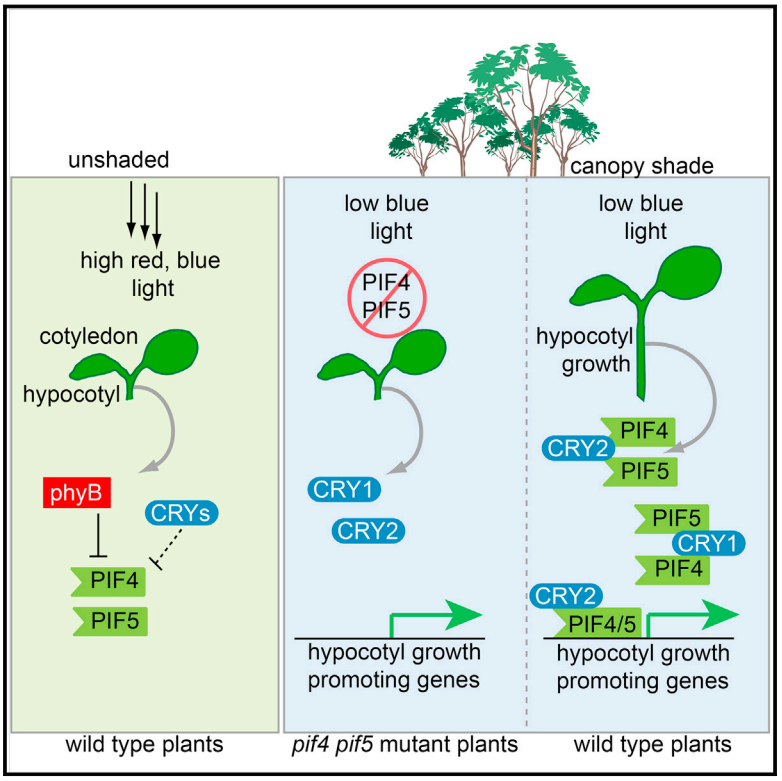
# Cryptochromes Interact Directly with PIFs to Control Plant Growth in Limiting Blue Light

## Graphical Abstract




## Highlights

- *Arabidopsis* CRY1 and CRY2 interact with phytochrome-interacting factors (PIF) 4 and 5
- Genes regulated under low blue light are distinct from those regulated in low red:far-red light
- CRY2 binds to common DNA regions shared with PIF4 and PIF5

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## In Brief

Plant cryptochromes respond to limiting blue light and regulate gene expression through modulating the activity of PIF transcription factors.

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# Cryptochromes Interact Directly with PIFs to Control Plant Growth in Limiting Blue Light

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## SUMMARY

**Sun-loving plants have the ability to detect and avoid shading through sensing of both blue and red light wavelengths. Higher plant cryptochromes (CRYs) control how plants modulate growth in response to changes in blue light. For growth under a canopy, where blue light is diminished, CRY1 and CRY2 perceive this change and respond by directly contacting two bHLH transcription factors, PIF4 and PIF5. These factors are also known to be controlled by phytochromes, the red/far-red photoreceptors; however, transcriptome analyses indicate that the gene regulatory programs induced by the different light wavelengths are distinct. Our results indicate that CRYs signal by modulating PIF activity genome wide and that these factors integrate binding of different plant photoreceptors to facilitate growth changes under different light conditions.**

## INTRODUCTION

Plants are incapable of long-distance migration and therefore must respond appropriately to ever-changing environmental challenges. Numerous environmental factors influence plant development, including temperature, light, touch, water, and gravity. Plants use this information to determine the time of day and season, to assess and anticipate temporal fluctuations in resource availability, and to determine whether they will soon be in the shade of another plant (Casal, 2012). Of the many environmental input pathways, light is understood the best. Light is perceived by a complex array of photoreceptors, which are defined by the color of light they absorb. In *Arabidopsis*, phytochromes (phyA–E) absorb red/far-red light; cryptochromes (CRY1–2), phototropins (phot1–2), and three ZTL-type receptors absorb blue/UV-A light; and one receptor (UVR8) absorbs UV-B light (Casal, 2012; Chory, 2010). Combinatorial analysis of these 13 photoreceptor genes indicate that they have complex synergistic, antagonistic, and redundant relationships (Mazzella and Casal, 2001). Photoreceptor signaling pathways primarily regulate the expression of a large number of genes involved in light responses, which then lead to the differential growth of specific cells and organs within the plant.

Plants have developed adaptive responses to detect and interpret changes in light quantity, quality, and directionality. In sun-loving plants, one such adaptive response is the ability to sense and avoid shading by neighboring plants. To avoid shading, plants utilize a suite of responses that are collectively known as the shade avoidance response (SAR) (Smith, 1982). During SAR, energy resources are re-allocated from storage organs to enable the rapid elongation of stems and petioles (leaf-stalk), allowing a shaded plant to outcompete its neighbors. Prolonged exposure to shade is costly. Shaded plants have accelerated reproductive development, leading to lower biomass and seed yield (Casal, 2012).

SAR is triggered not only by a reduction in total amount of visible light (400–700 nm; known as photosynthetically active radiation [PAR]) but also changes in spectral composition (Ballaré, 1999; Smith, 1982). Photosynthetic pigments within the plants absorb most of the visible light (red and blue), whereas far-red (FR) light is poorly absorbed and most is transmitted through or reflected by leaves. For instance, the red to far-red ratio (R:FR) of light is ~1.15 during midday and varies little with the weather or season. However, R:FR can be as low as 0.09 underneath a vegetational canopy (Smith, 1982). Underneath a canopy, there are simultaneous reductions in red (low R:FR) and blue light, and plants likely monitor these spectra to evaluate shading. Low R:FR has long been associated with all shade avoidance responses; however, studies in a variety of species and settings have shown that reduced or low blue light (LBL) can also invoke shade avoidance responses. Seedlings grown at a constant fluence rate of red light, but specifically deprived of blue light, resemble in many ways plants exposed to low R:FR (Ballaré, 1999; Keller et al., 2011; Keuskamp et al., 2010b).

A mechanism for how phytochromes (PHYs) regulate shade-induced growth in response to low R:FR light has been proposed. The detection of low R:FR by PHYs leads to the dephosphorylation of phytochrome interacting factor (PIF7), a bHLH TF that directly interacts with phyB (Li et al., 2012). This allows PIF7 to bind E-boxes in the promoters of a subset of *YUCCA* genes that encode the rate-limiting enzymes in auxin biosynthesis. This leads to increased levels of auxin, upregulation of auxin-regulated genes, and transport (Keuskamp et al., 2010a; Tao et al., 2008). When grown in low R:FR, loss-of-function mutations in *TAA1* or *PIF7* do not upregulate auxin levels and therefore do not exhibit SAR (Li et al., 2012). Thus, a role for the newly synthesized auxin that is both temporally and spatially distributed in early shade avoidance responses has been well documented.

In contrast, the molecular mechanisms by which the CRYs regulate hypocotyl growth in response to the LBL canopy signal (Bouly et al., 2007; Pierik et al., 2009; Sellaro et al., 2010) are less clear. Several phytohormones have been implicated in LBL-induced elongation responses, including auxin (Keuskamp et al., 2011; Pierik et al., 2009). However, auxin transport inhibitors that block low R:FR-induced elongation do not completely block hypocotyl elongation in response to LBL, indicating that R:FR and LBL growth responses maybe mediated by separate mechanisms (Keuskamp et al., 2011). Here, we demonstrate that the bHLH transcription factors (TFs) PIF4 and PIF5 are necessary for LBL-induced hypocotyl growth and that they act downstream of CRY1 and CRY2. We also show that CRY1 and CRY2 physically interact with PIF4 and PIF5. Transcriptome analysis indicates that the LBL response does not involve over-representation of auxin-regulated genes. Finally, we show by chromatin immunoprecipitation sequencing (ChIP-seq) that PIF4/5 and CRY2 occupy overlapping promoter regions, indicating that CRYs signal by modulating PIF4 and PIF5 activities to promote growth under LBL. As PIF4/5 act downstream of CRYs and PHYs to transduce blue- and red-light signals, respectively, these proteins represent a molecular basis for cross talk between these photosensory pathways. This work provides mechanistic detail to our understanding of how CRYs regulate plant growth.

## RESULTS

### PIF4 and PIF5 Mediate Hypocotyl Elongation in Response to LBL

The bHLH TFs, PIF1, 3, 4, 5, and 7 directly link PHYs to light-regulated gene expression and growth. PIF4 also plays a role in sensing high temperatures, which also cause rapid extension of the hypocotyl (Koini et al., 2009). We therefore tested whether PIFs also act downstream of CRYs to mediate hypocotyl growth in responses to LBL. We grew various *pif* mutants in white light (WL) for 5 days and then moved them to LBL for 4 days (Figure 1A). In contrast to wild-type (WT) seedlings, the hypocotyls of *pif4* and *pif5* seedlings did not elongate in response to LBL (Figures 1B, 1C, and S1A). In similar experiments, *pif4* and *pif5* seedlings exhibited appropriate hypocotyl elongation in response to low R:FR (Figure 1C), indicating that PIF4 and PIF5 predominantly mediate responses to LBL. However, *pif7* mutant seedlings, which fail to elongate under low R:FR (Li et al., 2012), responded partially to LBL (Figure 1D). We then measured hypocotyl length of *pif* quadruple (*pif1pif3pif4pif5*/*pifQ*) mutant seedlings (Leivar and Monte, 2014) and found that they failed to respond to LBL but clearly elongated in response to low R:FR, albeit to a lesser extent than WT (Figure 1D), suggesting that PIF1 and PIF3 do not play an important role in this process. To determine whether PIF4/5 act in the same genetic pathway as CRYs, we generated *cry1pif4* double and *cry1pif4pif5* triple mutants. These hypocotyls failed to elongate in LBL (Figures 1E and S1D), whereas *cry1* mutant showed a slightly exaggerated hypocotyl growth response compared to WT consistent with previous findings (Pierik et al., 2009). Using the HyDE technique to measure new hypocotyl growth (Cole et al., 2011), we found that the initiation of hypocotyl elongation response in LBL was slower than the response to low R:FR (Figure S1B). In addition, hypocotyl growth inhibition during de-etiolation under monochromatic blue light was pronounced in *cry1pif4* and *cry1pif4pif5* seedlings compared to *cry1* single-mutant hypocotyls (Figure S1E). We also constructed transgenic lines overexpressing PIF4 (*PIF4ox*) under the constitutive CaMV 35S promoter in the *cry1* mutant background. Two independent lines showed increased hypocotyl elongation under LBL conditions compared to the *cry1* (Figure 1F). Together, these results indicate that PIF4 and PIF5 function downstream of CRYs to mediate hypocotyl elongation in response to LBL.

### PIF4 and PIF5 Proteins Respond Dynamically to LBL

Many reports have shown that light destabilizes PIFs (with the exception of PIF7) and that the accumulation of PIF proteins correlates with their function (Leivar and Monte, 2014). We investigated the effect of LBL on PIF4 and PIF5 protein levels by growing PIF4/5-Flash (expressed under 35S promoter) seedlings and then exposing them to LBL for various durations. Western blotting revealed the accumulation of PIF5-Flash protein within 1 hr of LBL exposure, whereas PIF4-Flash protein levels remained constant (Figure 2A). In contrast, PIF5-Flash protein only transiently accumulated under low R:FR, peaking at 2 hr of exposure and declining back to baseline levels by 16–24 hr (Figure S2A). Because light fluence levels regulate CRY2 protein levels (Yu et al., 2007), we examined the dynamics of CRY1 and CRY2 protein accumulation in WT under LBL. CRY1 and CRY2 levels increased marginally in response to LBL (Figure 2B). In contrast, phyA and phyB protein levels were not affected by LBL exposure. Expression of *CRY1* and *CRY2* transcripts increased after 6 or 24 hr of LBL exposure compared to seedlings grown in WL (Figure 2C). However, these increases in expression were independent of PIF4 or PIF5, as similar *CRY1* and *CRY2* expression increases were seen in *pif4pif5* seedlings exposed to LBL (Figure 2C). To spatially visualize PIF4 and PIF5 protein accumulation in response to LBL, we fused PIF4/5 coding sequences to β-glucuronidase (GUS) and drove expression of these reporter genes via their native promoters (Figure S2B). Five-day-old seedlings stably expressing these transgenes were treated with LBL for up to 96 hr and then subjected to histochemical staining. We observed broad distributions of PIF4-GUS and PIF5-GUS throughout the seedlings when they were treated with LBL for <24 hr (Figures 2D and 2E). After 24 hr, PIF4 and PIF5 accumulated in the apical portion of the hypocotyl

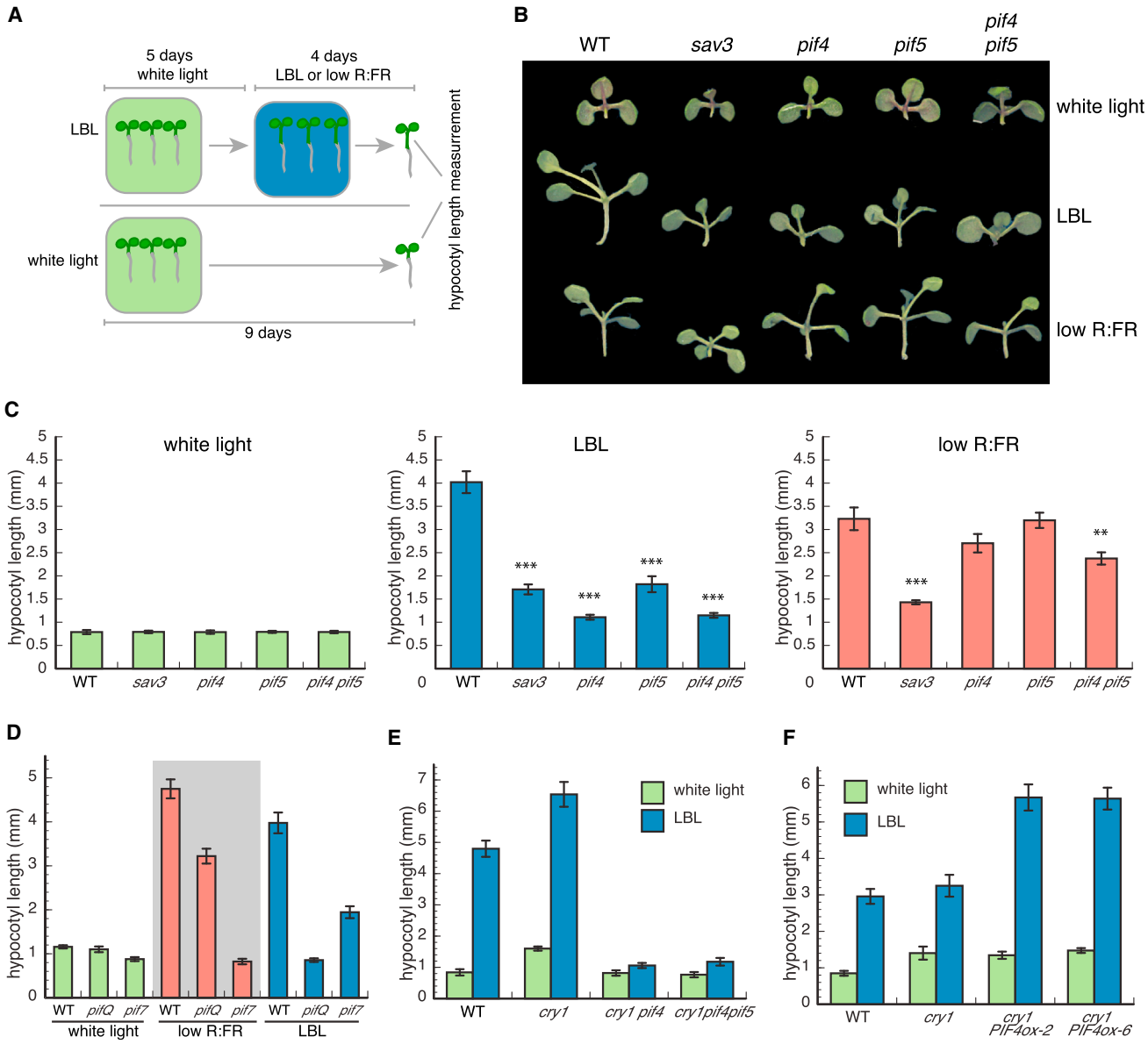


**Figure 1. PIF4 and PIF5 Mediate Hypocotyl Elongation in Response to LBL**

(A) Schematic diagram to assay hypocotyl length in LBL compared to a WL control. 5-day-old seedlings grown in continuous WL were treated with LBL or low R:FR or mock treated for 4 days prior to hypocotyl measurement. See also Figure S1C.

(B) Comparison of hypocotyl phenotypes of indicated seedlings in different light conditions.

(C) Hypocotyl length measurements after the indicated treatments (***$p < 0.001$ and **$p < 0.01$; Student's t test).

(D) Hypocotyl length measurements of WT, *pif7*, and *pifQ* (*pif1pif3pif4pif5*).

(E) Hypocotyl length of WT, *cry1*, *cry1pif4*, and *cry1pif4pif5* seedlings in different light conditions.

(F) Hypocotyl length measurements of *PIF4* overexpressors in *cry1* after LBL treatment.

Error bars indicate SEM. See also Figure S1.

and were absent from the basal region. In *Arabidopsis*, cell elongation occurs from the base of the hypocotyl (Boron and Vissenberg, 2014), potentially indicating that PIF4 and PIF5 are present in cells of the hypocotyl and the petioles that are actively elongating under LBL. Under low R:FR conditions, however, PIF4-GUS and PIF5-GUS proteins accumulated in all cells of the hypocotyl (Figures S2C and S2D).

## PIF4 and PIF5 Interact Directly with CRY1 and CRY2

In mouse, the bHLH TF BMAL1 interacts with CRY1/2 (Langmesser et al., 2008). Similarly, in *Arabidopsis*, the bHLH TF CIB1 interacts with CRY2 (Liu et al., 2008). In *Arabidopsis*, CRY2 is present only in the nucleus, whereas CRY1 is present in the nucleus and cytoplasm (Yu et al., 2007). PIFs are primarily nuclear (Leivar and Monte, 2014). To investigate

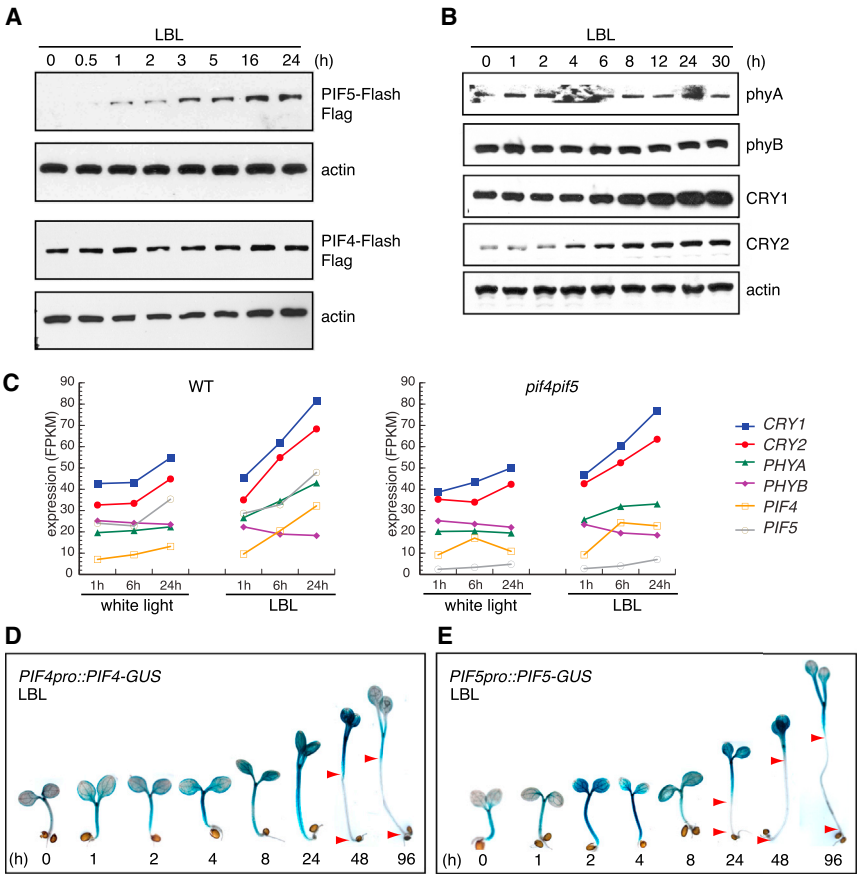


**Figure 2. Expression and Accumulation Dynamics of PIF4 and PIF5**

(A) 5-day old seedlings expressing PIF4/5-Flash proteins under the 35S promoter were exposed to LBL for indicated durations (hr). Western blots of protein extracts are shown. Flash-tagged proteins were detected using anti-Flag antibody. Actin served as the loading control.

(B) WT seedlings were treated as above. Western blots detecting phyA, phyB, CRY1, and CRY2 are shown. Actin served as the loading control.

(C) Normalized mRNA expression (FPKM) for indicated genes. 5-day-old seedlings were grown in WL for 5 days and then exposed to LBL or WL for indicated durations (hr). Total RNA samples were then collected and subjected to whole transcriptome RNA-seq.

(D and E) Histochemical detection of PIF4/5-GUS proteins driven using their native promoters. 5-day-old seedlings were exposed to LBL for indicated durations (hr) and processed to visualize GUS reporter activity. Regions between the arrowheads are devoid of GUS activity, as determined by visual inspection.

See also Figure S2.

whether *Arabidopsis* CRYs interact with PIF4 and PIF5, we performed a bimolecular fluorescence complementation (BiFC) assay. We tagged CRY1 and CRY2 with the amino-terminal portion of yellow fluorescent protein (nYFP) and tagged PIF4 and PIF5 with the carboxyl terminus of YFP (cYFP). When co-expressed, we observed a strong fluorescence signal, evident of YFP reconstitution, in nuclei of plants treated with LBL for 12 hr (Figures 3A and S3A). This indicates that these proteins can interact in planta and that the interaction occurs in the nucleus. We observed similar nuclear-localized interactions between phyB and PIF4, which served as a positive control (Figure S3B).

To confirm these results, we co-immunoprecipitated HA-PIF4 and Flag-PIF5 with Myc-CRY1/2 under light (Figures 3B and 3C), demonstrating their potential for physical interaction in vitro. CRY1-PIF5 interaction was not detected in the dark, whereas CRY2-PIF5 interaction was independent of light (Figure S3C). We also tested the interaction between CRYs and PIFs in HEK293 cells—a heterologous system devoid of PHYs. This is important because phyA binds CRY1 and phyB binds CRY2 (Más et al., 2000), which could confound our analyses. When HA-PIF4 and HA-PIF5 were expressed in HEK293 cells, they co-immunoprecipitated with *Arabidopsis* Myc-CRY2 in light (Figure 3D). CRYs have two distinct conserved domains (Figure 3E), the amino-terminal chromophore binding PHR (photolyase homology region) and the carboxyl-terminal CCE (cryptochrome C-terminal extension) domain (Liu et al., 2008). The Myc-tagged PHR domain of CRY2 interacted with full-length Flag-PIF5 (Figure 3F), whereas the N-terminal region of PIF5 (Flag-PIF5-N) interacted with Myc-CRY2 (Figure 3G). PIFs contain a conserved motif called active phytochrome binding (APB), which is necessary for binding to phyB (Figure S3D) (Khanna et al., 2004). To determine the possibility that CRY-PIF interaction occurs via the APB motif, we tested the binding between Myc-CRY2 and Flag-PIF5 containing mutated APB motif ($PIF5^{mAPB}$). Interaction between $PIF5^{mAPB}$ and CRY2 was not eliminated (Figure S3E) and, as reported, $PIF5^{mAPB}$ did not interact with the active Pfr form of phyB (Figure S3D). This finding indicates that CRY2-PIF5 contact occurs outside the APB motif. In addition, we demonstrated the CRY-PIF interaction in planta under LBL using co-immunoprecipitation using transgenic lines expressing mCitrine-CRY1 and mCitrine-CRY2, along with PIF4-Flash and PIF5-Flash. After exposing these transgenic lines to 16 hr of LBL, mCitrine-CRY1/2 immunoprecipitated PIF4/5-Flash proteins (Figures 3H and 3I). Together, these results imply that CRY1 and CRY2 physically interact with PIF4 and PIF5.

## phyB Plays a Role in LBL Responses

Since we have implicated PIF4/5 in responses to LBL, we sought to determine whether phyB also mediates LBL responses. We first measured hypocotyl elongation responses to LBL for transgenic lines expressing phyB under its own promoter (*PHYBpro::PHYB-Cit*), as well as for lines overexpressing phyB (*35Spro::PHYB-CFP*). Surprisingly, seedlings overexpressing phyB did not elongate in response to LBL (Figure 4A) compared to WT

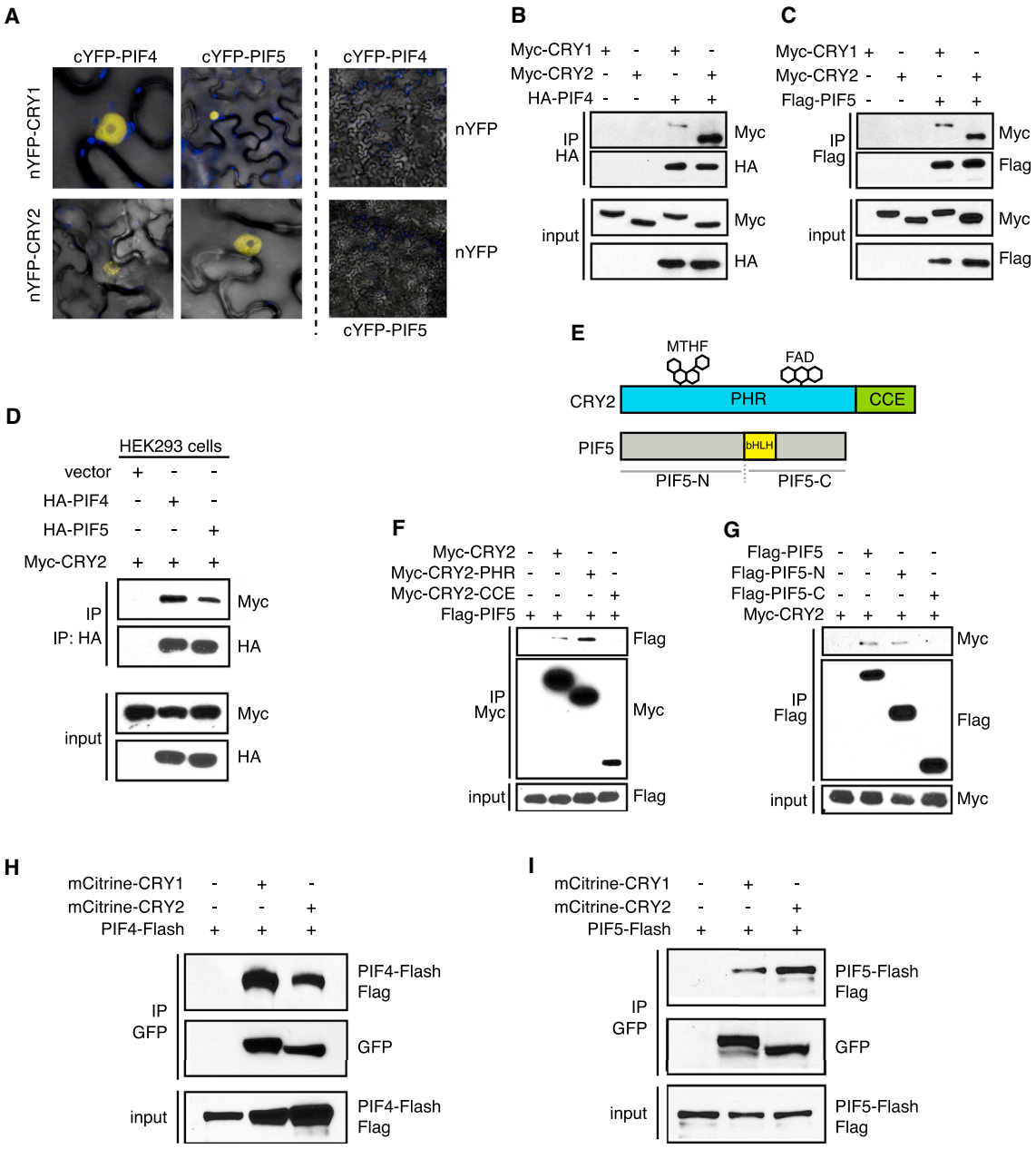


**Figure 3. PIF4 and PIF5 Interact with CRY1 and CRY2**

(A) CRY1 and CRY2 (tagged with nYFP) interact with PIF4 and PIF5 (tagged with cYFP) in *N. benthamiana* as assayed by BiFC. Blue signal denotes auto-fluorescence from chloroplasts.

(B and C) Myc-CRY1 and Myc-CRY2 interact with HA-PIF4 and Flag-PIF5 in vitro. Proteins were synthesized in a cell-free system, and co-immunoprecipitation was performed under light.

(D) Myc-CRY2 interacts with HA-PIF4/5 in human embryonic kidney (HEK293) cells. Post 72 hr of transfection, co-immunoprecipitation was performed under light.

(E) Schematic diagram showing conserved domains in *Arabidopsis* CRY2 and PIF5 proteins. MTHF, 5-methyltetrahydrofolate; FAD, flavin adenine dinucleotide; bHLH, basic helix-loop-helix; PHR, photolyase homology region; and CCE, cryptochrome C-terminal extension.

(F) In vitro co-immunoprecipitation of Flag-PIF5 with Myc-tagged PHR and CCE domains of CRY2. Experiments were performed as in (B).

(G) In vitro co-immunoprecipitation of Myc-CRY2 with Flag-tagged amino (N)- and carboxyl (C)-terminal portions of PIF5. Experiments were performed as in (B).

(H and I) PIF4/5-Flash protein co-immunoprecipitates with mCitrine-CRY1/2 from 5-day-old seedlings exposed to LBL for 16 hr.

See also Figures S3.

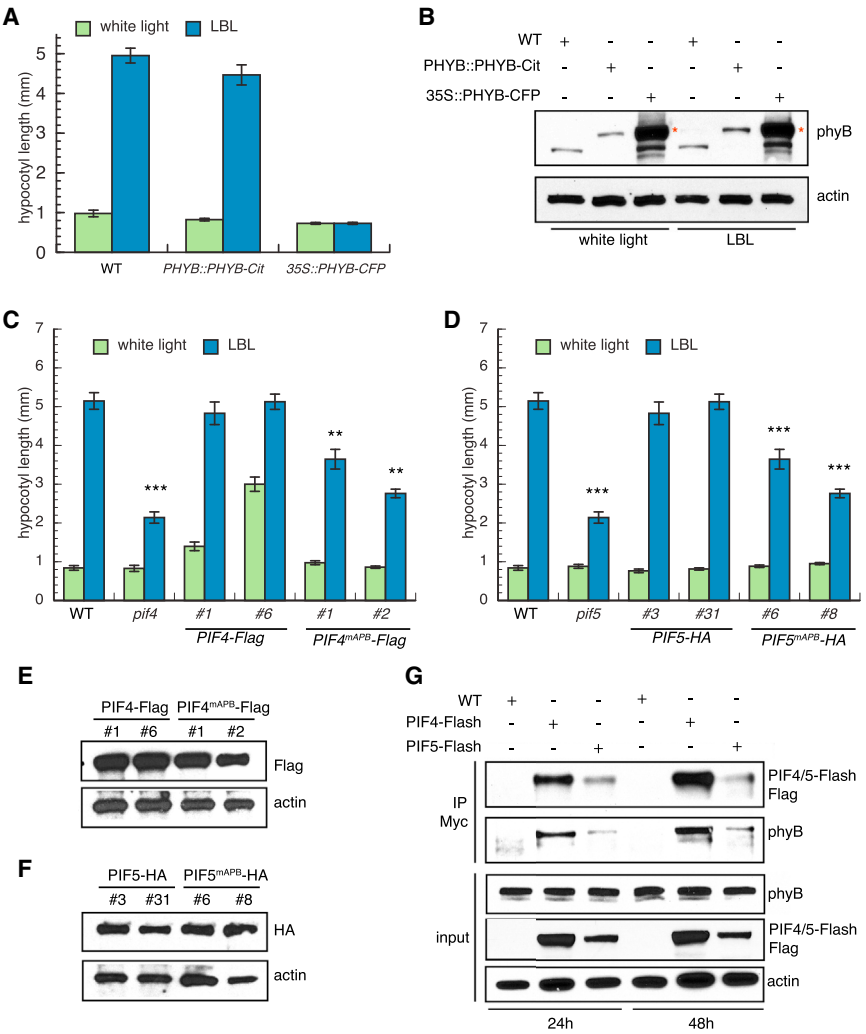


**Figure 4. phyB Plays a Role in LBL Responses**

(A) Hypocotyl length measurements of indicated genotypes.

(B) 5-day-old WT seedlings grown in WL for 5 days were exposed to LBL or mock treated for 24 hr. Levels of phyB protein were assessed using western blotting. Asterisk denotes expected band and actin served as the loading control.

(C) Hypocotyl length measurements of WT, *pif4* mutants, or *pif4* mutants expressing *PIF4-Flag* or *PIF4$^{mAPB}$-Flag* via *PIF4* promoter. (***$p < 0.001$ and **$p < 0.01$; Student's t test). Numerals with the pound sign represent independent lines.

(D) Experiments described in (C) were repeated for PIF5 (the *PIF5* promoter was used to drive transgene expression). Quantitation of hypocotyl length is shown. (***$p < 0.001$; Student's t test). Numerals with the pound sign represent independent lines.

(E and F) PIF4-Flag, PIF5-HA, PIF4$^{mAPB}$-Flag, and PIF5$^{mAPB}$-HA expressing seedlings described in (C) and (D) were used to detect the indicated proteins. Seedlings were grown in WL for 5 days and then exposed to LBL for 6 hr. Actin served as the loading control.

(G) Seedlings expressing PIF4/5-Flash were grown in WL for 5 days and then exposed to LBL for 24 or 48 hr. Co-immunoprecipitation was performed using an anti-Myc antibody that detects Flash-tagged proteins; phyB was detected using an anti-phyB antibody. Actin served as the loading control. Error bars indicate SEM.

and seedlings expressing phyB under its own promoter. This suggests that extremely high levels of phyB suppress LBL responses. However, phyB protein levels in these transgenic and WT plants did not significantly change in response to LBL (Figure 4B). Since phyB protein levels were extremely high in the phyB overexpression line, we hypothesize that phyB was sequestering PIF4/5, thereby preventing them from promoting growth and interacting with CRYs. We also mutated the conserved APB motif of PIF4 and PIF5 (PIF4/5$^{mAPB}$), rendering them unable to interact with phyB (Khanna et al., 2004). We generated transgenic plants expressing PIF4/5$^{WT}$ and PIF4/5$^{mAPB}$ under their own promoters in *pif4* or *pif5* loss-of-function mutant backgrounds. Multiple lines expressing PIF4$^{mAPB}$ only partially rescued the *pif4* mutant phenotype in response to LBL (Figure 4C). Similar results were seen with PIF5 (Figure 4D). In contrast, PIF4$^{WT}$ and PIF5$^{WT}$ completely rescued the *pif4* and *pif5* mutant phenotypes, respectively. The inability to fully elongate in response to LBL by PIF4/5$^{mAPB}$ cannot be explained by differences in protein levels (Figures 4E and 4F), clearly implicating the phyB-PIF interaction (via the APB domain) as critical for the LBL response. To next determine whether phyB complexes with PIF4/5 in LBL, we grew seedlings expressing PIF4/5-Flash proteins in WL for 5 days and then exposed them to LBL for 24 and 48 hr. Co-immunoprecipitation revealed that phyB was associated with PIF4 and PIF5 in LBL (Figure 4G). Furthermore, previous studies have shown that phyB promotes the inactivation or degradation of PIF proteins in certain light conditions (Ni et al., 2014; Park et al., 2012). Our results indicate that, in some cases, as in LBL, phyB-PIF interaction has a positive role. Taken together, these results indicate that phyB plays an active and important role in mediating cellular responses to LBL.

## LBL and Low R:FR Elicit Different Changes in Global Gene Expression

To further understand the genes regulated by PIF4 and PIF5 in response to LBL, we performed whole-genome mRNA-seq analysis. Five-day-old WT or *pif4pif5* double-mutant seedlings were placed into LBL or WL, and then samples were collected at three time points (1, 6, and 24 hr; Figures 5A and S4A). We found that the genes regulated by LBL in WT seedlings were misregulated in *pif4pif5* seedlings (Figure S4B and Tables S1 and S2). De novo discovery of enriched motifs associated with these misregulated genes (taking into account the 6 and 24 hr time points) identified "[CG]ACGTG[TG][CA]" (E-value 7.2E–21) and "CACGTG[TG][CAT]" (E-value 1.3E–14) as the top-scoring motifs (Figure 5B). These are variants of the E-box, to which bHLH

proteins typically bind. The top recurring gene ontology (GO) terms (ranked by false discovery rate $\leq$ 0.05) among the misregulated genes were: cell wall modification and organization, cell wall biogenesis, cell growth, and water responses (Figure 5C). The genes associated with these GO terms predominantly encoded expansins, extensins, and xyloglucan endotransglucosylase/hydrolase (XTH) cell-wall-modifying proteins (Table S3). Expansins regulate cell wall enlargement in growing cells, whereas XTHs promote wall loosening and strengthening of cell walls (Cosgrove et al., 2002). Extensins mediate cell wall self-assembly and extension in growing cells (Kieliszewski and Lamport, 1994). A large number of genes belonging to these families were differentially expressed between WT and *pif4pif5* seedlings, particularly after 6 hr of LBL (Figure 5D). Previous reports suggested that these cell-wall-modifying genes are direct targets of PIF1, 3, 4, and 5 (Hornitschek et al., 2012; Zhang et al., 2013) and that several members of the XTH gene family are upregulated in LBL (Keuskamp et al., 2011).

Exposure of seedlings to either LBL or low R:FR leads to hypocotyl elongation (Figure 1). We next determined whether these light conditions elicit similar changes in gene expression. In WT seedlings, we compared genes upregulated in response to LBL (data generated in this study) to those upregulated in response to 1 hr of low R:FR (publicly available RNA-seq datasets; Li et al., 2012). We found stark differences between the two transcriptome datasets with very little overlap, with Pearson's correlation $R$ = 0.125, 0.024 and −0.062 for 1, 6 and 24 hr in LBL, respectively (Figures 5E and S4C). In addition, some of the well-known low R:FR marker genes (Cole et al., 2011) were not induced in LBL (Figure S5A). These results indicate that LBL and low R:FR affect different sets of target genes. Published genome-wide transcriptome analyses have repeatedly shown that low R:FR induces predominantly the expression of auxin responsive genes (Li et al., 2012; Sessa et al., 2005). We compared 335 known auxin-induced genes between our LBL transcriptome data to the previously described low R:FR dataset (Li et al., 2012). However, in LBL, the WT seedlings did not regulate the same auxin-responsive genes (Figure 5F). We measured free auxin (IAA) levels in WT, *pif4pif5*, *PIF4-Flash*, and *PIF5-Flash* whole seedlings exposed to LBL for 1 or 24 hr. We observed no changes in free auxin levels across these genotypes and treatments (Figure S5B). This observation likely explains why auxin-induced genes are not affected by LBL (Figure 5F). Under low PAR conditions, upregulation of the auxin receptor AFB1 is thought to confer increased auxin sensitivity when auxin levels do not change (Hersch et al., 2014). However, for WT and *pif4pif5* seedlings grown in LBL, expression levels of *AFB1* (log2 fold increase in WT = 0.21) and other auxin-receptors were unaffected (compared to white-light controls; Figure S5C). Additionally, we find that seedlings carrying triple mutations in *PIN* auxin efflux transport family members (*pin3pin4pin7*) had hypocotyl elongation defects in LBL (Figure S5D); in contrast, a single mutation in *pin3* led to similar defects in low R:FR (Keuskamp et al., 2010a). Our results further reinforce that hormonal action differs in plants exposed to LBL in comparison to low R:FR and suggest that PIF4 and PIF5 control hypocotyl elongation in LBL by largely regulating cell-wall-modifying proteins.

### PIF4, PIF5, and CRY2 Bind to Common Chromatin Regions

We hypothesized that, since PIF4 and PIF5 mediate responses to LBL and bind to CRYs in vivo, CRYs could physically associate with genomic regions associated with PIF targets. To test this, we performed ChIP-seq experiments on Flash-CRY2, PIF4-Flash, and PIF5-Flash seedlings treated with LBL for 16 hr. Using stringent peak-calling criteria, we identified a large number of overlap between PIF4, PIF5, and CRY2 target genes (Figure 6A and Table S4). De novo discovery of enriched motifs within the PIF4 and PIF5 binding peaks identified the E-box variant "CA[CT]GTG" (E-value 8.9E–408) and "CA[CT]GTG" (E-value 1.5E–398) as a top-scoring motif (Figure 6B). Similarly, the E-box variant "C[AG]CGTG" (E-value 2.2E–95) was enriched in CRY2 peaks (Figure 6B). Next, we compared CRY2 ChIP signals with PIF4/5 ChIP signals, and we discovered that CRY2 peaks significantly overlap with PIF4/5 peaks (Figures 6C and S6A), and CRY2 binding is reproducibly enriched at PIF4/5 peaks (Figures 6D and S6B). This analysis was verified using ChIP-qPCR, where we saw overlap between PIF4- and CRY2-enriched genomic regions surrounding the E-box motif within the previously characterized *HFR1* chromosomal region (Figure S6C) (Hornitschek et al., 2009). Out of the 30 to 50% PIF4, PIF5, CRY2, and their shared targets differentially expressed under LBL (Figure S6D), we found similar levels of misregulation in *pif4pif5* background for the PIF4, PIF5, PIF4+CRY2, and PIF5+CRY2 target gene sets (Figure 6E). In addition, the top enriched GO terms were shared between the PIF4, PIF5, CRY2, PIF4+CRY2, and PIF5+CRY2 bound genes (Figure S6E). Overall, these results suggest that PIF4/5 and CRY2 are present at the same genomic loci and regulate similar genes in LBL.

To understand the significance of binding of CRY2 and PIF4/5 to various promoter regions of genes in LBL, we investigated the role of genes in the hypocotyl growth response, whose expression in LBL increased as well as bound by CRY2 or jointly with PIF4/5. We identified several such genes meeting these criteria, and we analyzed two genes—*ATHB2* (a homeobox leucine zipper protein; bound by PIF4 and 5 and CRY2) and *XTH33 (*Xyloglucan endotransglucosylase/hydrolase; bound by CRY2)—in detail. Overexpression of *XTH33* and *ATHB2* showed exaggerated hypocotyl growth when compared to WT specifically under LBL (Figure 6F). Furthermore, it has been reported that overexpression of several other target genes—*BBX21*, *EBF1*, and *EBF2* (bound by PIF4 and 5 and CRY2)—results in a longer hypocotyl (Crocco et al., 2010; Guo and Ecker, 2003). These observations suggest that CRY2 regulates gene expression upon binding to PIF4/5 transcription factor to regulate hypocotyl growth in LBL.

## DISCUSSION

The mechanisms by which the final body plans of multicellular organisms are established have mostly been elusive. Plants are sessile, and their organs emerge post-embryonically, making them ideal for studying multicellularity, growth, and the control of organ size. The final shape and size of genetically identical plants can vary substantially, as each organism adopts a morphology that is optimal for their particular environment (Depuydt and

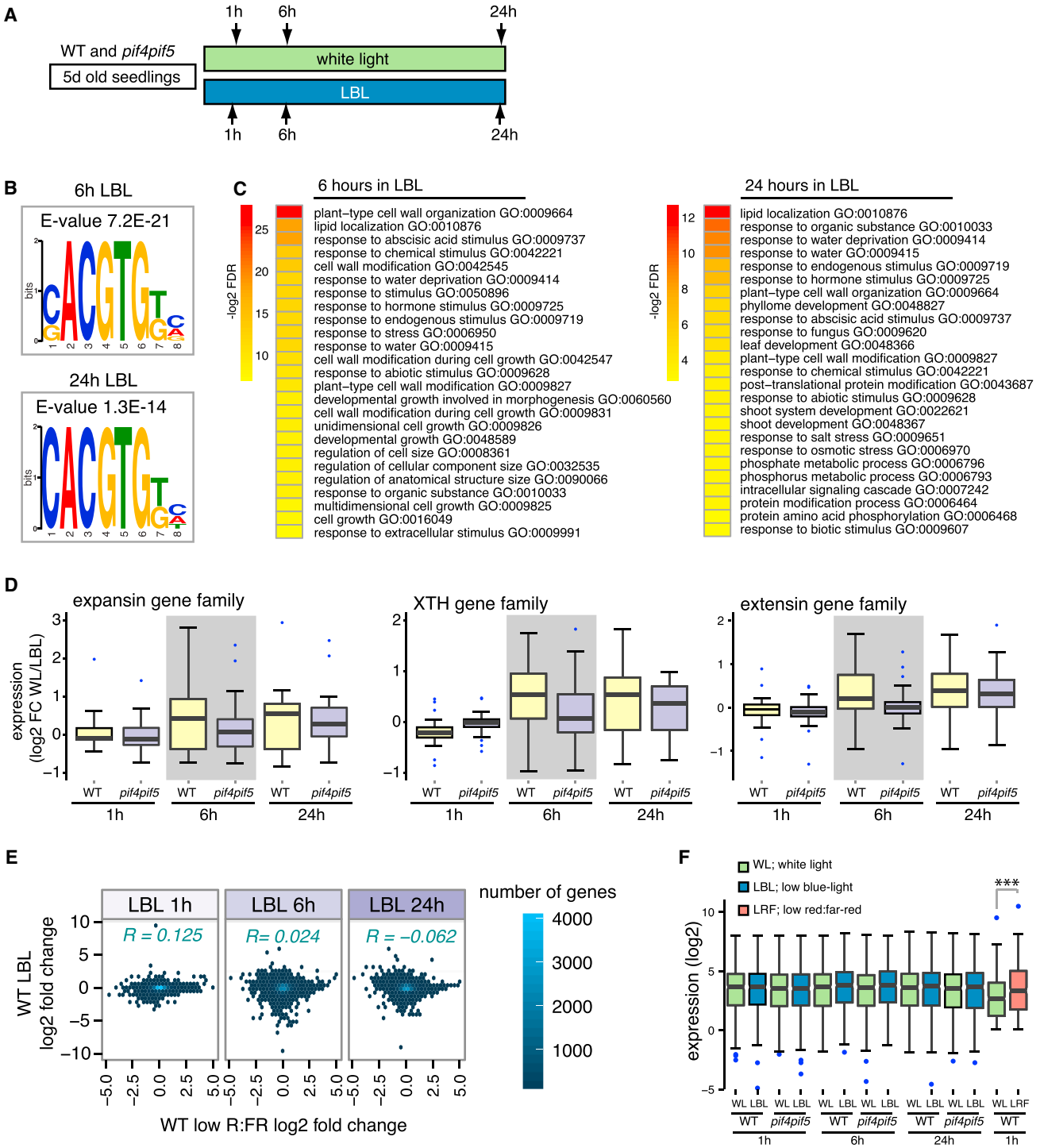


**Figure 5. Transcriptome Changes in LBL Are Distinct from Low R:FR**

(A) Experimental scheme for the mRNA-seq analysis on WT and *pif4pif5* mutant seedlings in LBL compared to WL.

(B) Top de novo enriched motifs in the promoter region of genes that were misregulated in *pif4pif5* seedlings compared to WT in response to LBL. Motifs associated with 6 and 24 hr of LBL exposure are shown. No significant motifs were identified for the 1 hr time point.

(C) Top 25 (ranked by false discovery rate [≤0.05]) significantly enriched GO terms derived from genes that are misregulated in *pif4pif5* seedlings compared to WT after 6 or 24 hr of LBL. No significant GO terms were identified for the 1 hr time point.

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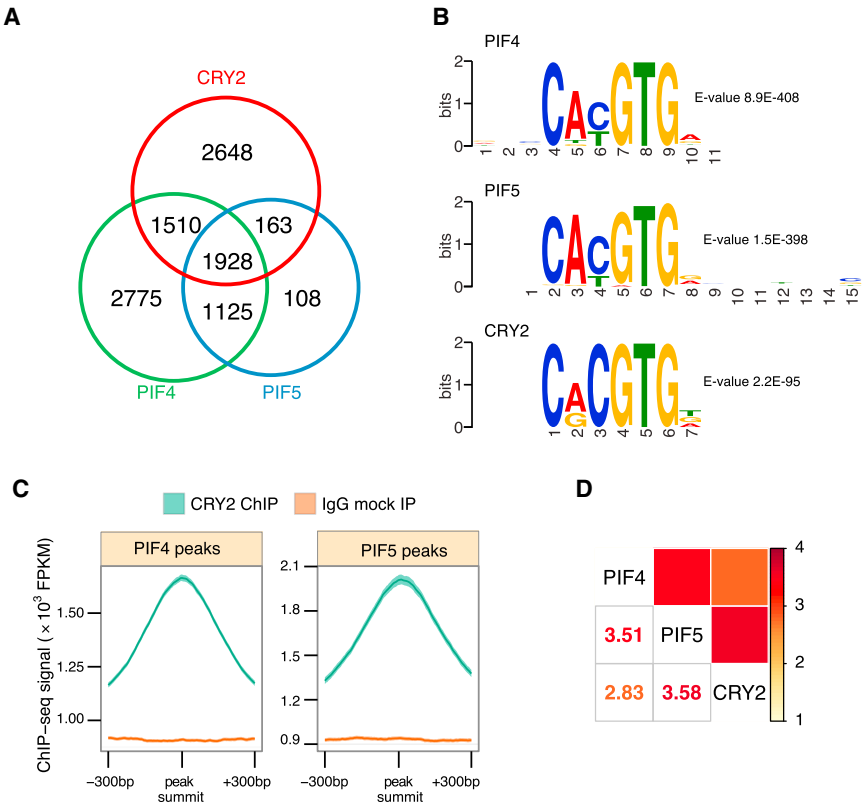


**Figure 6. PIF4, PIF5, and CRY2 Bind to Common Genomic Regions**

(A) Venn diagram depicting the overlap between PIF4, PIF5, and CRY2 target genes determined by ChIP-seq.

(B) De novo enriched motifs identified within 200 bp surrounding peak summits of PIF4, PIF5, and CRY2 ChIP-seq peaks with ≥3-fold enrichment in LBL.

(C) Average CRY2 ChIP-seq signals surrounding PIF4 and PIF5 binding peak summits (±300 bp is shown). PIF4/5 IDR optimal peaks, and ≥2-fold enrichment was used. Shaded areas represent the 95% confidence interval.

(D) Log2 fold enrichment of pairwise association between PIF4, PIF5, and CRY2 peaks compared to shuffled control. All p values are ≤ 0.001 by 1,000 randomizations.

(E) PIF4, PIF5 bound genes and PIF4/5 and CRY2 common genes that are differentially expressed under LBL show similar level of misregulation in *pif4pif5* seedlings.

(F) Hypocotyl length measurements of indicated genotypes. 5-day-old seedlings grown in continuous WL were treated with LBL or mock treated for 3 days prior to hypocotyl measurement. Error bars indicate SEM. (***p < 0.001 and *p < 0.05; Student's t test.)

See also Figure S6 and Table S4.

Hardtke, 2011). SAR of sun-loving plants is an excellent example of this plasticity. SAR is a rapid response that includes quantitative increases and decreases in growth rates of different organs (Casal, 2012).

PHYs and CRYs detect and mediate responses to red/far-red light and blue/UV-A light, respectively. These receptors are broadly expressed in plant tissues and have overlapping and distinct roles in environment-mediated growth and development (Tóth et al., 2001). PhyA physically interacts with CRY1, and phyB binds CRY2 (Más et al., 2000). Here, we show that CRYs also physically interact with PIFs, which are downstream signaling partners of PHYs. CRYs and PHYs both regulate flowering but in different cell types—CRY2 in the vascular bundles and phyB in the mesophyll cells (Endo et al., 2007, 2005). Light is also known to regulate tissue-autonomous promotion of palisade cell development by phot2 (Kozuka et al., 2011). These examples and our study further underscore the importance of examining spatial and temporal changes in gene expression, protein levels/localization, and metabolism to understand how growth is regulated within specific cells and tissues, especially when signaling components are shared. As such, only limited conclusions can be drawn from the many published studies because they only examine phenotypic endpoints. Future studies should make an attempt to separate cell-type-specific responses.

(D) Indicated gene families (involved in cell wall modification and enlargement) are expressed at higher levels in WT compared to *pif4pif5* seedlings in response to LBL. Center lines are the medians; box limits indicate the 25th and 75th percentiles, whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; outliers are represented by blue dots.

(E) 2D Histogram of log2 fold changes in gene expression in response to LBL (y axis) compared to log2 fold changes in response to low R:FR (x axis; data from Li et al., 2012). Data for 1, 6, or 24 hr of LBL exposure are shown as separate panels. Each point represents one gene and "*R*" indicates Pearson's correlation.

(F) Box plot representation of 335 auxin-induced genes for WT and *pif4pif5* seedlings exposed to indicated light and durations. (***p < 0.001; ANOVA followed by Tukey's test).

See also Figures S4 and S5 and Tables S1, S2, and S3.

To date, PIF1, 3, 4, 5, and 7 have been shown to bind phyB, while PIF1 and 3 also bind phyA (Leivar and Monte, 2014). Some of these PIFs are functionally redundant, but there are reports of their independent roles. For example, specific PIFs mediate growth responses to LBL (PIF4 and PIF5), low R:FR (PIF7), high temperature (PIF4), and freezing tolerance (PIF4 and PIF7) (Koini et al., 2009; Lee and Thomashow, 2012; Li et al., 2012). In addition, because PIFs are bHLH TFs, they bind E-boxes as both homo- and hetero-dimers, and they also bind other TFs such as DELLAs (Oh et al., 2014). As different heterodimers of bHLH TFs can cause very distinct phenotypes during murine limb development (Firulli et al., 2007), it is conceivable that PIF4-PIF5 dimers or as heterodimers with other factors could dictate the functional outcome of the LBL response. It appears that PIFs are bound to DNA (at least in some conditions) when they interact with PHYs (Martínez-García et al., 2000), and our results suggest that CRY2 interacts with PIF4/5 when they are bound to chromatin. Since CRY2 and phyB contact PIF5 at distinct domains (Figures S3D and S3E), it is possible that these three types of proteins bind chromatin as a multimeric complex. Thus, in light of many possible combinations of protein-protein interactions, we suggest that the concentration of individual PIFs, CRYs, and PHYs dictates the output of the light response in a particular cell type. For instance, constitutive overexpression of CRY1 and CRY2 in all cells led to increased growth inhibition of the hypocotyl under monochromatic blue light (Lin et al., 1998), but selective increase in CRY protein levels is seen under LBL (Figure 2B). It is likely that, in LBL, CRYs accumulate in cells that promote growth, and it is tempting to speculate that CRYs could have opposite roles in different cells. Thus, the quantity of individual signaling components and molecular outputs in specific cells, coupled with knowledge of the affinities of individual PIFs for E-boxes within promoters of light-regulated genes, likely determines the organismal response.

We chose to study shade avoidance because it is a phenotypically simple, quantitative, and manipulatable growth response that involves changes in the cell expansion rate of hypocotyl cells by changes in light quality (low R:FR or LBL) or quantity (PAR). Recent studies have suggested that hypocotyl cell elongation is driven by a central growth-regulation program (e.g., Oh et al., 2014). To our surprise and contrary to these reports, our data indicate that hypocotyl elongation is driven by several parallel pathways acting independently in response to different environmental stimuli involving multiple photoreceptors acting in different tissues via different hormone pathways. While we agree that PIFs likely play a general role in growth under many conditions, we suggest that the upstream pathways acting on PIFs are diverse and condition specific rather than linear. During phyB-mediated shade avoidance, auxin and auxin-regulated genes are upregulated for growth (Keuskamp et al., 2010a). Low PAR is likely perceived by CRYs, phototropins, and PHYs, but the signal is transmitted via an unknown mechanism that involves an increased sensitivity to auxin, rather than the synthesis of new auxin (Hersch et al., 2014). We show here that depletion of blue light (LBL) triggers yet another growth pathway that does not involve changes in detectable auxin levels or sensitivity (Figure S7). These results argue that there is more than one pathway leading to growth of the hypocotyl in response to different colors or the amount of light.

Why are there multiple and redundant growth pathways in SAR? It is likely that, for a noisy and constantly changing environment like that present in a canopy (Keller et al., 2011), redundant systems are necessary to accurately process all the information provided by light. A plant does not want to initiate SAR if a cloud passes overhead or there is a flash of lightning in the middle of the night.

TFs can rapidly re-program the transcriptome to alter cellular architecture. It is intriguing to note that the downstream genes regulated by the CRYs and phyB are quite different despite involvement of the same bHLH TFs in these pathways (Figure 5). In low R:FR, 50% of the upregulated genes are genes known to be regulated by auxin (Sessa et al., 2005; Tao et al., 2008). In LBL, our transcriptome analysis showed that PIF4 and PIF5 largely controlled cell wall re-modeling and expansion genes (Figures 5C and 5D). We did not detect significant auxin-regulated transcripts in LBL (Figure 5F), although auxin generated by the TAA1/SAV3 enzyme and transported by PIN efflux transporters is required for full induction of hypocotyl elongation in LBL (Figures 1B, 1C, and S5D) (Keuskamp et al., 2011). It is possible that auxin production and distribution have different kinetics in a spatially and temporally distinct manner in LBL, and our current time points did not detect it. In our LBL conditions, there may be increased hormone sensitivity by a route of action different from that seen under low PAR and R:FR. A second paradox concerns the initial slow rate of hypocotyl elongation in LBL when compared to low R:FR (Figure S1B), even though their phenotypic end points are similar (Figure 1C). One might have predicted that the LBL pathway has faster kinetics than low R:FR, a pathway that seems to be more complex.

Light might control the activity of a CRY-PIF complex by regulating its protein level or by other modifications. CRY2 protein is degraded under high fluence rates of light (Yu et al., 2007), and likewise, PIF5 protein levels are low in WL (Figure 2A). Therefore, it can be anticipated that, under limiting blue light conditions (like LBL), PIF5 levels are high, which then leads to its interaction with CRY1/2 to promote growth. Red light is known to promote proteasomal degradation of PIF3/5 (Leivar and Monte, 2014). However, LBL treatment of seedlings caused accumulation of PIF5 protein, even though red light was present in our assays (Figure 2A). In LBL, CRYs may shield PIF5 from PHY-mediated proteasomal degradation, similar to observations in *Drosophila*, where CRY sequesters TIMELESS (Ceriani et al., 1999). In addition, our results suggest that PIF4/5 form the molecular basis of signal integration between CRY and PHY photoperception events. The loss of *PIF4* and *PIF5* in a *cry1* mutant background resulted in shorter hypocotyls in an additive manner during de-etiolation under monochromatic blue light. These results and previous observations (Kunihiro et al., 2010) suggest that CRYs promote de-etiolation by modulating PIF activity in blue light, just as PHYs modulate PIF activity to regulate hypocotyl length in red, far-red, and low R:FR light (Leivar and Monte, 2014; Lorrain et al., 2008).

Our results indicate that CRY2 likely localizes to chromatin indirectly, via associations with PIFs or other TFs, similar to how phyA associates with regulatory regions in the genome

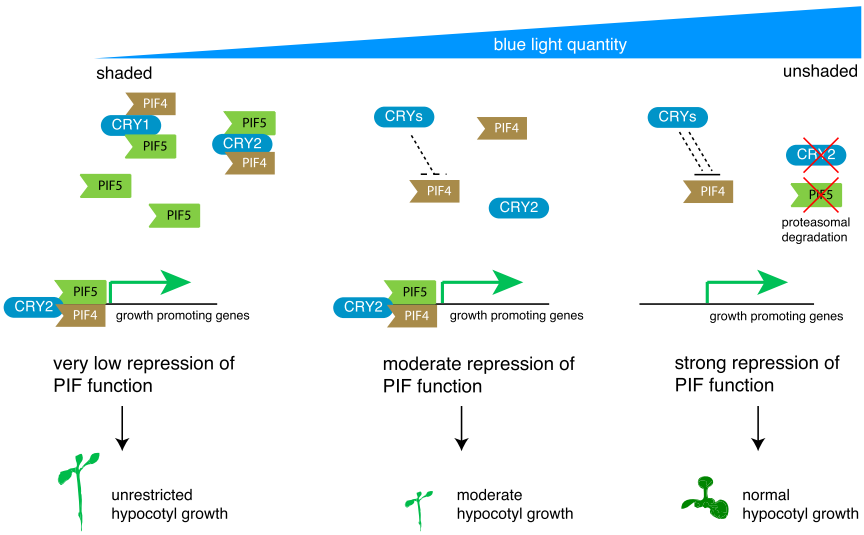


**Figure 7. Proposed Model on the Role of CRYs and PIF4/5 in Their Regulation of Hypocotyl Growth in a Changing Blue Light Environment**

In ample blue light conditions, concomitant negative regulation of PIF activity by CRYs and diminished levels of CRY2 and PIF5 proteins does not promote hypocotyl growth. Under moderate and low levels of blue light, there is an accumulation of PIF5 and CRY2 proteins; in addition, physical interactions between CRYs and PIF4/5 are facilitated. In LBL, CRY2 and PIF4/5 are associated with the promoters of the genes that promote growth. Therefore, plants rapidly fine-tune their growth strategy upon changes in the light environment by modulating PIF4/5 activity through CRY2 on the chromatin. *Arabidopsis* seedlings lacking CRY1 and CRY2 leads to a constitutive LBL response due to unchecked PIF activity leading to an unrestricted hypocotyl growth.

(Chen et al., 2014). We hypothesize that CRY1/2 and PIF4/5 likely interact on nuclear DNA and that photoactive, nuclear-localized PHYs could also be interacting with PIFs on the DNA. CRY2 binding to PIF factors could provide it with specificity to bind the promoter of a gene whose expression is required for the hypocotyl growth response. Therefore, we propose that CRY2 can affect transcription positively or negatively depending on the gene and physiological and developmental context of the plant. Results from our study suggest that CRYs directly regulate the activity of PIFs and other TFs through physical interactions on the DNA, such that changes in the external environment can rapidly lead to phenotypic changes (Figure 7).

The exact reasons why plants have evolved multiple ways to regulate the same growth process will remain unclear until additional data have been obtained. The presence of multiple growth-promoting pathways likely underlies the wide spectrum of phenotypic responses, as well as morphological variations seen in plants. A plethora of receptors, compound circuits, binding partners, cell/tissue types, and downstream components help plants respond and adapt to a wide variety of environmental stresses and growth conditions. Elucidating these growth control circuits will likely require experiments that take into account temporal and spatial factors within specific cell types.

## EXPERIMENTAL PROCEDURES

### Hypocotyl Length Measurements

All hypocotyl length assays were performed on seedlings grown on 0.5× Linsmaier and Skoog (LS; Caisson Laboratories) medium containing 0.8% phytagar (Caisson Laboratories). After indicated treatments, seedlings were placed horizontally on 0.5× LS medium and were imaged using a flat-bed scanner. Hypocotyl length was measured using ImageJ software (http://imagej.nih.gov/ij/).

### Protein Extraction and Immunoblotting

Proteins were resolved on Bis-Tris gels using MOPS running buffer (Invitrogen). Immunoblots were performed as described (Pedmale and Liscum, 2007). Antibodies used for the immunoblots as follows: CRY1 and CRY2 (C. Lin), phyA and phyB (A. Nagatani), Flag M2 (Sigma), Flag-HRP (Sigma), c-Myc 9E10 (Covance), c-Myc-HRP, and GFP-HRP (Miltenyi Biotec), GFP-HRP (Abcam), HA 3F10, and HA-HRP (Roche). Appropriate goat secondary antibodies conjugated to HRP were used (Bio-Rad).

### In planta Co-immunoprecipitation

Co-immunoprecipitation (co-IP) was performed as described (Nusinow et al., 2011) with modifications. Approximately 1–3 g of whole seedlings was transferred to 2 ml tubes with three stainless steel balls and then frozen in liquid nitrogen and disrupted in a bead beater. The ground tissue was resuspended in 800 μl SII buffer (100 mM sodium phosphate [pH 8], 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% Triton X-100, 1.5× protease inhibitors (Sigma), 75 μM MG132, 10 mM NaF) and sonicated at 10% power, with 0.5S on/off cycles for a total of 10 s on ice. Extracts were then clarified by centrifugation at 4°C for 10 min, measured for protein concentration using Bradford reagent (Bio-Rad), and normalized for western blots and co-IPs. For co-IPs, extracts of equal total proteins were mixed with Protein-G magnetic beads (Invitrogen) crosslinked with anti-Flag antibody (Nusinow et al., 2011) or anti-GFP paramagnetic beads (Miltenyi Biotec) for 1 hr with gentle rotation at 4°C. The beads were washed 3× with 1 ml of SII buffer. Precipitated proteins were eluted by heating beads at 70°C for 5 min in 25 μl of 2× SDS-PAGE loading buffer.

## ACCESSION NUMBERS

Next-generation DNA sequencing raw and processed data has been deposited into the Gene Expression Omnibus (GEO) with accession numbers GEO: GSE59699 and GEO: GSE68193.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.12.018.

## AUTHOR CONTRIBUTIONS

U.V.P. and J.C. designed the experiments, and U.V.P. performed most of the experiments with the following exceptions: S.C.H. performed RNA-seq and ChIP-seq analyses, P.A.B.R. performed the ChIP-qPCR analysis, J.H. performed the BiFC experiments and phenotypic measurements, B.J.C. contributed qRT-PCR and HyDE analyses, K.L. measured free IAA, P.S. expressed proteins in mammalian cells, M.Z. performed ChIP-seq experiments, J.R.N. performed sequencing and K.N. provided reagents. J.R.E. supervised S.C.H. and M.Z. All authors commented on the manuscript.

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