Direct photoresponsive inhibition of a p53-like transcription activation domain in PIF3 by *Arabidopsis* phytochrome B

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Photoactivated phytochrome B (PHYB) binds to antagonistically acting PHYTOCHROME-INTERACTING transcription FACTORS (PIFs) to regulate hundreds of light responsive genes in *Arabidopsis* by promoting PIF degradation. However, whether PHYB directly controls the transactivation activity of PIFs remains ambiguous. Here we show that the prototypic PIF, PIF3, possesses a p53-like transcription activation domain (AD) consisting of a hydrophobic activator motif flanked by acidic residues. A PIF3mAD mutant, in which the activator motif is replaced with alanines, fails to activate PIF3 target genes in *Arabidopsis*, validating the functions of the PIF3 AD in vivo. Intriguingly, the N-terminal photosensory module of PHYB binds immediately adjacent to the PIF3 AD to repress PIF3’s transactivation activity, demonstrating a novel PHYB signaling mechanism through direct interference of the transactivation activity of PIF3. Our findings indicate that PHYB, likely also PHYA, controls the stability and activity of PIFs via structurally separable dual signaling mechanisms.
The plant genome is exquisitely sensitive to and can be transcriptionally reprogrammed by changes in light intensity and composition. In particular, alterations in the intensity and ratio of red (R, 600–700 nm) and far-red (FR, 700–750 nm) light are powerful environmental cues that inform about space and time—such as the availability of photosynthetically active R-light radiation, the sense of morning and evening as well as seasonal fluctuations in day length, and the threat by neighboring vegetation or shade that depletes R-light even as well as seasonal synthetically active R-light radiation, the sense of morning and evening and of coordinated actions allowing seedlings to quickly emerge from the soil31,32. To promote hypocotyl elongation, PIFs bind to the enhancer regions of growth-relevant genes, such as those involved in the biosynthesis and signaling of the plant growth hormone auxin, and activate their transcription. Upon seedling emergence from the ground and exposure to light, photoactivated PHYB translocates to the nucleus and interacts with PIF1, PIF3, PIF4, and PIF5 to promote their rapid phosphorylation, ubiquitylation, and degradation by the proteasome, thereby reprogramming the expression of light-responsive PIF target genes19,33–35. PHYB-triggered degradation of PIFS has been considered a central mechanism of PHYB signaling26,27, implying that PHYB regulates PIF target genes mainly indirectly by controlling the abundance of PIFS.

Accumulating circumstantial evidence suggests that PHYB may also directly control the transcriptional activity of PIFS. First, not all PIFS are degraded in the light or by photoactivated PHYB. For example, the protein level of PIF7 is not significantly different between dark and light conditions36,37. Also, although PIF4 and PIF5 rapidly diminish during the initial exposure to light, when seedlings are grown under diurnal or continuous light conditions, both can accumulate to high levels during the daytime, when PHYB is active38,39. Moreover, the stability of PIFS varies at different temperatures. For example, PIF3, despite being almost undetectable in ambient temperatures, becomes stabilized in the light in cold temperatures40. Therefore, the PHYB-PIF interaction could regulate the activity of PIFS in light conditions. Second, although PHYB triggers the light-induced degradation of PIF3, recent studies surprisingly revealed that the light-dependent interaction between the light-sensing knot of PHYB and the ABP of PIF3 is not responsible for PIF3 degradation16,41,42. Instead, PIF3 degradation relies on the weaker, light-independent interaction with PHYB’s C-terminal output module16,41,42. These results leave open the important question of what the functional significance of the PHYB-ABP interaction is in PIF3 regulation. One proposed hypothesis is that binding of PHYB to the ABP regulates the transcriptional activity of PIFs41,43. Supporting this hypothesis, the PHYB-ABP interaction was shown to attenuate PIF3’s DNA-binding activity independently of its degradation41,43. However, because the APB motif is located at the very N-terminus of PIF3, far from the C-terminal bHLH domain, and PHYB does not interact directly with the bHLH24,27, it remains unclear whether PHYB influences the DNA binding of PIF3 directly or indirectly via the control of its transactivation activity.

The main roadblock that hinders our understanding of the relationship between PHYB and the transactivation activity of PIFS is the lack of a molecular understanding of PIFS’ transcription activation domains (ADS). It is well understood that transcriptional activators follow a modular design consisting of a DNA-binding domain to selectively bind to specific DNA sequences (enhancers) and an AD to initiate transcription44. Although the classes of DNA-binding domains have been well defined and can be recognized based on conserved amino acid sequences45, ADS remain difficult to predict due to their poor sequence conservation46–48. As a result, despite PIFS being widely accepted as transcriptional activators28, their AD sequences have not been precisely defined. A previous attempt to map the AD of PIF3 identified two separate regions in its N-terminal half that carry transactivation activity in yeast, although these putative ADS have not been validated in planta49. To further explore the possibility of a direct link between PHYB and the transactivation activity of PIFS, first, we utilized truncation analysis and alanine-scanning mutagenesis combined with yeast and in planta assays to determine the AD of PIF3. Our results show that PIF3 possesses a single AD, which surprisingly resembles the ADS of the mammalian tumor suppressor p53 and the yeast activator Gcn4, revealing the unexpected conservation of sequence-specific ADS across the animal, fungal, and plant kingdoms. We demonstrate that binding of PHYB’s N-terminal
photosensory module to the APB motif of PIF3 represses PIF3’s transactivation activity, revealing a new PHYB signaling mechanism via direct photo-inhibition of the transactivation activity of PIFs.

Results
The aa91-114 region of PIF3 confers AD activity in yeast. To define and validate the AD of PIF3, ideally, it requires evaluation of the in planta functions of a PIF3 mutant specifically lacking its AD activity while leaving other biochemical functions intact. A previous study identified two PIF3 regions with transactivation activity in yeast using a modified yeast one-hybrid screen and random mutagenesis. However, the PIF3 mutants isolated via this approach contained multiple mutations; therefore, the residues directly and specifically required for the AD activity could not be unambiguously pinpointed. To circumvent these potential issues, we chose to systematically map the region and residues that are both necessary and sufficient for PIF3’s transactivation activity by truncation analysis and targeted mutagenesis. Because PIF3 has been shown to be a potent activator in yeast, we used yeast transactivation assays to examine PIF3’s AD activity. We fused the Gal4 DNA-binding domain (DBD) to either the full-length PIF3 or a series of N- or C-terminal PIF3 truncation fragments in the bait construct of the Matchmaker Gold yeast two-hybrid system (Fig. 1a). We evaluated the self-activation activity of the recombinant DBD-PIF3 proteins using two assays: (1) a cell viability assay testing the activation of the Aureobasidin A (AbA) resistance gene AUR1-C and (2) a liquid assay quantifying the activation of a second reporter, β-galactosidase (Fig. 1a). Interestingly, all PIF3 fragments containing the region between amino acids 91 and 114 (aa91-114)—i.e., PIF3-N1 to PIF3-N4, as well as PIF3-C1 and PIF3-C2—retained at least 87% of the activity of full-length PIF3 (Fig. 1a). In contrast, the two fragments missing part of aa91-114—i.e., PIF3-N5 and PIF3-C3—completely lost the activity (Fig. 1a). The aa91-114 fragment alone (PIF3-M2) was sufficient to activate gene expression, albeit with reduced activity (Fig. 1a). The weaker activity of aa91-114 could be due to a lack of structural support provided by the adjacent protein context because a larger fragment containing amino acids 76–114 (PIF3-M1) displayed significantly higher activity (Fig. 1a).

Our results of aa91-114 being an activating region is consistent with the previous study, which showed that the slightly larger aa90-120 region carries transactivation activity. However, we did not detect any transactivation activity between amino acids 27 and 43, which encompass the second activating region suggested by the previous study. None of the four N-terminal fragments of PIF3 containing the second putative activating region, aa1-52, aa1-76, aa1-90, and aa1-101, showed any transactivation activity in our assays (Fig. 1a and Supplementary Fig. 1). To further resolve this discrepancy, we performed alanine-scanning mutagenesis in the region between amino acids 26 and 114 in the PIF3-N1 (aa1-331) fragment by substituting every five consecutive amino acids with five alanines (Fig. 1b). Corroborating the results of the truncation analysis, only the alanine substitution mutants in the aa91-114 region (m14–m18) completely abrogated the transactivation activity (Fig. 1b). In contrast, the mutants with mutations in the second suggested activating region (m1–m4) remained active, despite m1 and m2 showing reduced activities (Fig. 1b), indicating that the aa26-43 region is not essential for PIF3’s transactivation activity. Together, the results of the truncation analysis and alanine-scanning mutagenesis indicate that aa91-114 is the only region both required and sufficient for PIF3’s transactivation activity, and therefore, confers PIF3’s AD function in yeast.

PIF3 possesses a p53-like AD. Studies of animal and yeast activators suggest that one class of ADs comprises a short, sequence-specific activator motif of hydrophobic residues embedded in an intrinsically disordered region enriched in acidic residues. One identified activator motif is ΦxxΦΦ, where Φ indicates a bulky hydrophobic residue and x is any other amino acid. For example, both ADs in the mammalian tumor suppressor p53, AD1 and AD2, contain the ΦxxΦΦ motif. The same motif is also found in the yeast activator Gcn4. Surprisingly, aligning the PIF3 AD to the ADs of p53 and Gcn4 revealed striking similarities: PIF3’s AD features a FVPWL motif flanked by negatively charged aspartate and glutamate residues. The FVPWL motif in PIF3 shares the same hydrophobic residues as the activator motifs in p53 and Gcn4, including the two bulky aromatic amino acids F93 and W96. Both the activator motif and the negatively charged residues have been shown to play important roles in transactivation. For example, the ΦxxΦΦ motif in AD1 and AD2 of p53 mediates hydrophobic interactions with the transcriptional coactivator cyclic-AMP response-element-binding protein (CREB)-binding protein (CBP) and its paralog p300. The surrounding acidic residues participate in electrostatic interactions with transcriptional coactivators and create an intrinsically disordered context in the unbound state to enhance the accessibility of the activator motif. Both the ΦxxΦΦ motif and surrounding acidic residues are highly conserved in PIF3 orthologs in eudicots, supporting the idea that PIF3 possesses a p53-like AD.

To evaluate the contribution of the conserved residues around the ΦxxΦΦ motif to PIF3’s transactivation activity, we mutated amino acids 91 to 100 individually to alanine in the PIF3-N1 fragment. However, the single-amino-acid substitution mutants had little effect on PIF3’s AD activity (Supplementary Fig. 2a, b), suggesting that the main functional and/or structural attributes of the AD are fulfilled redundantly by multiple residues. We then replaced the five residues of the FVPWL motif with alanines (a combination that was not included in the alanine-scanning mutagenesis) in full-length PIF3 and named it PIF3mAD (Fig. 2b). PIF3mAD completely lost its transactivation activity, confirming that this is the only AD in PIF3 (Fig. 2c). In addition, replacing only the three key hydrophobic residues in the ΦxxΦΦ motif, namely F93, W96, and I97, with arginine or serine also abolished AD activity (Supplementary Fig. 2a, c); similar mutations were shown to impede the activity of p53, suggesting that the crucial roles of these hydrophobic residues in AD activity are conserved in PIF3.

The AD region of PIF3 was previously recognized as a motif of unknown function, which is moderately conserved among PIF family members (Fig. 2b). The ΦxxΦΦ motif can be recognized in PIF1, PIF4, PIF5, PIF7, and PIF8. While PIF1 and PIF8 contain a perfectly composed ΦxxΦΦ motif, PIF4, PIF5, and PIF7 are missing the initial hydrophobic residue (Fig. 2b). The flanking acidic residues are conserved to various degrees among PIF1, PIF4, PIF5, PIF7, and PIF8. In PIF1, PIF4, and PIF5, multiple acidic residues are present on both sides of the ΦxxΦΦ motif, whereas, in PIF7 and PIF8, at least one side of the ΦxxΦΦ motif contains significantly fewer acidic residues (Fig. 2b). The attributes of the PIF3 AD are least conserved in PIL1/PIF2 and PIL2/PIF6, in which the core motif is barely recognizable and the ΦxxΦΦ motif is completely lacking its hydrophobic residues. The Flavin protein PIF3 possesses a p53-like AD. Studies of animal and yeast activators suggest that one class of ADs comprises a short, sequence-specific activator motif of hydrophobic residues embedded in an intrinsically disordered region enriched in acidic residues. One identified activator motif is ΦxxΦΦ, where Φ indicates a bulky hydrophobic residue and x is any other amino acid. For example, both ADs in the mammalian tumor suppressor p53, AD1 and AD2, contain the ΦxxΦΦ motif. The same motif is also found in the yeast activator Gcn4. Surprisingly, aligning the PIF3 AD to the ADs of p53 and Gcn4 revealed striking similarities: PIF3’s AD features a FVPWL motif flanked by negatively charged aspartate and glutamate residues. The FVPWL motif in PIF3 shares the same hydrophobic residues as the activator motifs in p53 and Gcn4, including the two bulky aromatic amino acids F93 and W96. Both the activator motif and the negatively charged residues have been shown to play important roles in transactivation. For example, the ΦxxΦΦ motif in AD1 and AD2 of p53 mediates hydrophobic interactions with the transcriptional coactivator cyclic-AMP response-element-binding protein (CREB)-binding protein (CBP) and its paralog p300. The surrounding acidic residues participate in electrostatic interactions with transcriptional coactivators and create an intrinsically disordered context in the unbound state to enhance the accessibility of the activator motif. Both the ΦxxΦΦ motif and surrounding acidic residues are highly conserved in PIF3 orthologs in eudicots, supporting the idea that PIF3 possesses a p53-like AD.

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The aa91-114 region confers PIF3's AD activity in yeast. Full-length and a series of truncation fragments of PIF3 were fused to the Gal4 DNA binding domain (DBD) as shown in the schematics, using the bait vector of the yeast two-hybrid system, and were evaluated for their transactivation activity in yeast. The magenta column highlights the aa91-114 region. APB, active PHYB binding motif; APA, active PHYA binding motif.

Identification of the residues necessary for PIF3's AD activity via alanine scanning mutagenesis. A series of alanine-scanning mutants (m1–m18) were generated between amino acids 26 and 114 (indicated by the blue line) in the PIF3-N1 construct. The range of the alanine-substituted amino acids for each mutant is shown in parentheses. The mutants that lost their transactivation activity are shown in magenta.

The middle panel shows serial dilutions of the yeast strains containing the respective constructs grown on either SD/-Trp/+AbA or SD/-Trp (control) media. The right panel shows the relative transactivation activities quantified using the yeast liquid β-galactosidase assay. The transactivation activities were calculated relative to that of either PIF3 (a) or PIF3-N1 (b). DBD alone was used as a negative control. Error bars represent the s.d. of three biological replicates; the centers of the error bars represent the mean values. The numbers represent the mean value of the relative activity. The statistical significance of the changes between the sample and the control, PIF3 in a or PIF3-N1 in b, was analyzed by one-way ANOVA (Dunnett's test, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001, n.s. indicates no significant difference). The source data underlying the yeast liquid β-galactosidase assays in (a) and (b) are provided in the Source data file.
clear transactivation activity (Fig. 2b, c). PIF1, PIF4, and PIF5 were the most potent activators, with significantly higher transactivation activity than PIF3, whereas PIF7 and PIF8 were less active than PIF3 (Fig. 2c). Conversely, PIL1 and PIL2, which show little similarity to the PIF3 AD in the corresponding region, had the least transactivation activity; PIL1 in particular showed no detectable activity (Fig. 2b, c). Interestingly, PIL1 has been suggested to heterodimerize with PIF4, PIF5, and PIF7 to negatively regulate their activities. Therefore, the activity of the PIF family members might reflect their functional roles in PHY signaling.

To examine whether the ΦxxΦΦ motif contributes to the transactivation activities of PIF3 paralogs, we replaced the corresponding ΦxxΦΦ residues in PIF1, PIF4, PIF5, PIF7, and PIF8 with five alanines to create their respective mAD mutants. Intriguingly, only PIF7mAD lost its transactivation activity, suggesting the ΦxxΦΦ region in PIF7 is its sole AD (Fig. 2c). In contrast, mutating the ΦxxΦΦ motif did not abolish the activity of PIF1, PIF4, PIF5, or PIF8. PIF1mAD, PIF4mAD, and PIF8mAD remained active but had dramatically reduced activities compared with their respective wild-type proteins; the activity of PIF5 was barely affected by the mAD mutations (Fig. 2c). These results suggest that either the ΦxxΦΦ sequences in PIF1, PIF4, PIF5, and PIF8 are not essential for their AD activity or these PIFs contain one or more additional ADs.
perfectly conserved among PIF family members, suggesting a possible divergence in the mechanisms of transactivation among PIF members. These differences warrant further in-depth investigations on the AD functions of each individual PIF; we therefore focus the rest of the study on PIF3.

**PIF3mAD still binds PHYA and PHYB.** The AD of PIF3 lies between the APB and APA motifs (Fig. 1a)[34]. Next, we used in vitro GST pulldown assays to test whether the alanine mutations in PIF3mAD had an effect on PIF3 binding to PHYB and PHYA. Similar to GST-PIF3, GST-PIF3mAD could preferentially bind to the active Pr forms of hemagglutinin (HA)-tagged PHYB and PHYA without a detectable change in affinity in the pull-down assays (Fig. 3). These results indicate that the FVPWL activator motif of PIF3 does not directly engage in the interactions with PHYA and PHYB.

**PIF3mAD is impaired in PIF3’s functions in skotomorphogenesis.** To validate the PIF3 AD in vivo, we generated transgenic lines expressing HA- and YFP-tagged PIF3 (HA-YFP-PIF3) or PIF3mAD (HA-YFP-PIF3mAD) under the native PIF3 promoter as described previously[56]. The transactivation activity of PIF3 was best demonstrated during skotomorphogenesis. In dark-grown Arabidopsis seedlings, PIF3 binds to the enhancer regions of its target genes, and subsequently activates the expression of these genes, leading to the etiolated phenotype observed in the absence of light. However, skotomorphogenesis is maintained redundantly by three additional PIF members, PIF1, PIF4, and PIF5, which hetero-dimerize or hetero-oligomerize with PIF3[21,28,30,58]. Therefore, the potential effects of PIF3mAD could be obscured in the presence of the other three PIFs. To circumvent this redundancy issue, we generated transgenic lines expressing HA-YFP-PIF3 or HA-YFP-PIF3mAD in a pif1pif3pif4pif5 quadruple mutant (pifq)[29]. For each construct, we picked two transgenic lines expressing similar levels of HA-YFP-PIF3 or HA-YFP-PIF3mAD. Despite being controlled by the native PIF3 promoter, the tagged PIF3 proteins in all four transgenic lines were expressed at higher levels than was endogenous PIF3 in the wild-type Col-0 and the pifl45 triple mutant (Fig. 4a). We therefore assessed the activity of HA-YFP-PIF3mAD mainly via comparison with that of HA-YFP-PIF3.

Dark-grown pifq seedlings display de-etiolated phenotypes with short hypocotyls compared with Col-0 (Fig. 4b, c)[29,30]. The two transgenic lines expressing HA-YFP-PIF3 (PIF3/pifq 1-2 and 9-5) fully rescued the short-hypocotyl phenotype of pifq and had the same hypocotyl length as pif145, which expresses only PIF3 among the four PIFs (Fig. 4b, c). In contrast, the two lines expressing HA-YFP-PIF3mAD (PIF3mAD/pifq 2-1 and 4-5) only partially reverted the hypocotyl phenotype of pifq and remained significantly shorter than Col-0, pif145, and the two PIF3/pifq lines (Fig. 4b, c). HA-YFP-PIF3 and HA-YFP-PIF3mAD were expressed at similar levels in the transgenic lines, despite slightly less abundance of HA-YFP-PIF3mAD in the PIF3mAD/pifq lines (Fig. 4a). Both HA-YFP-PIF3 and HA-YFP-PIF3mAD localized to the nucleus (Fig. 4d). Therefore, the discrepancy in hypocotyl length between the PIF3/pifq and PIF3mAD/pifq lines was most likely due to the defect in the transactivation activity of HA-YFP-PIF3mAD. Indeed, the steady-state transcript levels of four PIF3 target genes[28], PIL1, ATHB-2, XTR7, and RD20, were significantly lower in the PIF3mAD/pifq lines than in the PIF3/pifq lines (Fig. 4e). Both the PIF3mAD/pifq and PIF3/pifq lines contained much higher levels of HA-YFP-tagged PIF3 compared with the endogenous PIF3 level in pif145 (Fig. 4a), however, the PIF3 target genes were mostly expressed at higher levels in the PIF3/pifq lines but at lower levels in the PIF3mAD/pifq lines compared with pif145 (Fig. 4e). To exclude the less-likely possibility that the reduced expression of the PIF3 target genes in the PIF3mAD/pifq lines was due to a defect of PIF3mAD in DNA binding, we examined the binding of HA-YFP-PIF3 and HA-YFP-PIF3mAD to the G-box elements in the enhancer region of the marker gene PIL1 via chromatin immunoprecipitation (ChIP). The results of the ChIP experiments confirmed that the mutations in PIF3mAD did not affect its DNA binding activity (Fig. 4f). Together, these results support the conclusion that PIF3mAD is impaired in the transactivation activity of PIF3 and provide strong in vivo evidence validating the aa91-114 region as PIF3’s AD. The fact that the PIF3mAD/pifq lines could still partially reverse the hypocotyl phenotype of pifq (Fig. 4b, c) suggests that either the PIF3mAD mutations do not completely abolish the AD activity in vivo, or alternatively, PIF3 can exert its functions through other associated transcription activators[56]. Supporting the latter possibility, genome-wide studies found that PIF-binding sites frequently coincide with binding sites of other families of transcription factors, some of which have been shown to directly interact with PIFs[59,60].

**PIF3 AD induces ELIP2 activation by light.** PIF3 activates not only light-repressed genes but also, surprisingly, some light-inducible genes when dark-grown seedlings are exposed to light for the first time. For example, PIF3 participates in the light-
dependent induction of **ELIP2** (**EARLY LIGHT-INDUCED PROTEIN 2**), which encodes a special chlorophyll-binding protein that protects the plastids during initial exposure to light radiation\(^56,61\). However, it was unclear whether **ELIP2** induction relies on **PIF3**’s intrinsic AD activity or the activity of other **PIF3**-associated transcription factors\(^56\). The **PIF3mAD** mutant therefore provides an opportunity to clarify the role of **PIF3**’s intrinsic activity in **ELIP2** induction. To that end, first, we examined the involvement of the **PIF3** AD in the early light-induced dynamics of **PIF3** subnuclear distribution, phosphorylation, and abundance. During the dark-to-light transition, **PHYA** and **PHYB** recruit **PIF3** to many small nuclear bodies and promote **PIF3**’s phosphorylation and subsequent degradation\(^62,63\). Because **PIF3mAD** retains the binding activities to active **PHYA** and **PHYB** (Fig. 3c), as expected, **HA-YFP-PIF3mAD** showed the same functionalities as **HA-YFP-PIF3** in light-dependent nuclear body localization, phosphorylation, and degradation (Fig. 5a, b). However, in striking contrast, the **PIF3mAD/pifq** lines failed to activate **ELIP2** as effectively as did the **PIF3/pifq** lines (Fig. 5c). Within 1 h of R light exposure, the **PIF3/pifq** lines elevated the transcript level of **ELIP2** by more than 100 times, whereas the transcript level of **ELIP2** increased 4-to-5-fold less in the **PIF3mAD/pifq** lines (Fig. 5c). These results indicate that **ELIP2** activation is mainly induced by **PIF3**’s intrinsic AD activity. Notably, **ELIP2** transcripts were also elevated significantly by light in **pifq**, albeit to a lesser extent (Fig. 5c), indicative of the involvement of other transcription factors in **ELIP2** induction besides the four **PIFs**. Therefore, **PIF3** may promote the full induction of **ELIP2** through both its own AD as...
Fig. 4 PIF3mAD compromises PIF3's functions in skotomorphogenesis. a Immunoblots showing the steady-state levels of endogenous PIF3, or HA-YFP-PIF3 and HA-YFP-PIF3mAD in dark-grown seedlings of Col-0, pifq, pif45, PIF3/pifq (1-2 and 9-5), and PIF3mAD/pifq (2-1 and 4-5) using anti-PIF3 antibodies. RN6 was used as a loading control. The numbers beneath the PIF3 immunoblot indicate the relative levels of PIF3 or HA-YFP tagged PIF3 normalized to RN6. The asterisk indicates non-specific bands. The immunoblot experiments were independently repeated at least three times, and the results of one representative experiment are shown. b Images of representative 4-d-old dark-grown seedlings of Col-0, pifq, and the transgenic PIF3/pifq and PIF3mAD/pifq lines. c Hypocotyl length measurements of the lines shown in (b). The boxes represent from the 25th to 75th percentile; the bars are equal to the median values; the whiskers extend to the maximum and minimum data points. Samples labeled with different letters exhibited statistically significant differences (ANOVA, Tukey’s HSD, P ≤ 0.05). d Confocal images showing the nuclear localization of HA-YFP-PIF3 and HA-YFP-PIF3mAD in hypocotyl epidermal cells of 4-d-old dark-grown PIF3/pifq (line 1-2) and PIF3mAD/pifq (line 2-1) seedlings. Nuclei were stained with DAPI. Scale bars represent 5 μm. e qRT-PCR results showing the steady-state transcript levels of select PIF3 target genes in seedlings described in (b). The transcript levels were calculated relative to those of PPA2. Error bars represent the s.d. of three biological replicates. The centers of the error bars represent the mean values. Numbers indicate fold changes relative to pifq; the statistical significance was analyzed using two-tailed Student’s t-test (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001). f ChIP assays showing the binding of HA-YFP-PIF3 and HA-YFP-PIF3mAD to the G-box elements in the enhancer region of PIL1, as illustrated in the schemas. The ChIP experiments were performed using 4-d-old dark-grown PIF3/pifq (line 1-2) and PIF3mAD/pifq (line 2-1) seedlings with anti-HA antibodies. Immunoprecipitated DNA was quantified via real-time PCR using primer sets for locations 1 to 6 at the PIL1 locus. ChIP with rabbit IgG was used as a negative control. Error bars represent the s.d. of three biological replicates. The centers of the error bars represent the mean values. The statistical significance was analyzed using two-tailed Student’s t-test. n.s. indicates either the values between the PIF3/pifq and PIF3mAD/pifq samples are not significantly different or the difference is <1.5-fold. The source data underlying the immunoblots in (a), hypocotyl measurements in (b), qRT-PCR data in (e), and ChIP data in (f) are provided in the Source data file.

PIF3 AD participates in gene activation by shade. PIF3 plays an important role in the rapid gene activation when PHYB is inactivated by shade or FR light. To assess the function of the PIF3 AD in the activation of shade-inducible genes during the light-to-shade transition, first, we characterized PIF3/pifq and PIF3mAD/pifq lines grown in continuous R light. Interestingly, while pifq shows a short-hypocotyl phenotype in the light, the slow growth of pifq was fully rescued in the PIF3/pifq lines (Fig. 6a, b). Under R light conditions, endogenous PIF3 does not accumulate to a detectable level. However, like the previously reported PIF3 transgenic lines, the two PIF3/pifq lines accumulated a significant amount of HA-YFP-PIF3 in the light (Fig. 6c), providing an explanation for their enhanced hypocotyl elongation in the light. On the other hand, the PIF3mAD/pifq lines only partially reversed the dwarf phenotype of pifq, and they were significantly shorter than the PIF3/pifq lines (Fig. 6a, b). HA-YFP-PIF3mAD accumulated to similar levels as HA-YFP-PIF3 (Fig. 6c), but the transcript levels of three PIF3-target genes, PIL1, ATHB-2, and IAA29, in PIF3mAD/pifq were similar to those in pifq and failed to increase to the levels in Col-0 and PIF3/pifq (Fig. 6d and Supplementary Fig. 3). Next, we treated the R-light-grown seedlings with 1 h of FR light (simulated shade) and then examined the expression of the same three PIF target genes before and after the FR treatment. The PIF3/pifq lines were able to dramatically enhance the expression of PIF3 target genes as Col-0 (Fig. 6d). However, like pifq, the PIF3mAD/pifq lines failed to activate PIF3 target genes to the extent as those in Col-0 (Fig. 6d), indicating that the PIF3 AD is required for the full activation of shade-inducible genes. It is important to note that our experimental design was not aimed at assessing the contribution of PIF3 in shade responses; because PIF3 accumulates to higher levels in our transgenic lines, the contribution of PIF3 to shade responses in our experimental settings was likely overestimated. However, our results clearly show that PIF3mAD is impaired in the transactivation activity under shade treatment.

Binding of PHYB and PHYA inhibits the transactivation activity of PIF3 and PIF1. In the shade experiments, we were intrigued by the fact that although HA-YFP-PIF3 accumulated to high levels in the PIF3/pifq lines (Fig. 6c), the transcript levels of the PIF3 target genes remained low in continuous R light (Fig. 6d). These observations suggest that the transactivation activity of HA-YFP-PIF3 must be repressed in the light by PHYB, and likely, this transcriptional repression is released by FR light upon PHYB inactivation. It has been postulated that PHYB inhibits PIF3 binding to the promoters of target genes. However, it remains ambiguous whether PHYB influences the DNA-binding activity of PIF3 directly or indirectly through the regulation of PIF3’s AD activity. Because PIF3 AD resides in close proximity to the APB motif, we hypothesized that binding of PHYB to the APB could directly inhibit the AD activity of PIF3 independently of its DNA-binding activity. To discern the AD activity of PIF3 from its DNA binding, we went back to the yeast transactivation assay and examined the activity of the PIF3-N4 fragment, which includes only the PIF3 AD and the APB motif without the bHLH DNA-binding domain (Fig. 7a). We coexpressed PIF3-N4 with PHYB conjugated with a phycocyanobilin (PCB) chromophore and performed yeast liquid β-galactosidase assays in either R or FR light to test whether the activity of PIF3-N4 could be influenced by the Pr or the Pr form of PHYB, respectively (Fig. 7a). We found that the Pr form of PHYB reduced the activity of PIF3-N4 by more than 70%, whereas the Pr form imposed little effect on PIF3-N4’s activity (Fig. 7b). Because PIF3 interacts with both PHYB’s N- and C-terminal modules, we then tested which interaction is responsible for the transcriptional inhibition. For the N-terminal module of PHYB, we adopted a previously demonstrated biologically active form called NGB, which combines the N-terminal module of PHYB with GFP and GUS as a dimerization domain. NGB was also able to inhibit the transactivation activity of PIF3-N4 in a light-dependent manner; in contrast, the C-terminal module of PHYB had little effect (Fig. 7b). These results demonstrate that the N-terminal photosensory module of PHYB can inhibit the transactivation activity of PIF3’s AD, and this activity of PHYB is independent of PIF3’s DNA-binding activity. Interestingly, PHYB and NGB failed to repress the activity of PIF3-M1, which contains the PIF3 AD but not the APB motif (Fig. 7c), supporting the notion that the photoinhibition of the PIF3 AD is mediated by a direct interaction between PHYB and the APB motif. Both PHYB and NGB could also repress the activity of PIF1 in a light-dependent manner (Fig. 7d), suggesting that PHYB can inhibit the activity of other PIFs via the same mechanism. Besides the APB motif, PIF3 and PIF1 contain the APA motif near the AD
Fig. 5 Characterization of PIF3mAD in early light signaling. a Confocal images showing the nuclear body localization of HA-YFP-PIF3 and HA-YFP-PIF3mAD in the hypocotyl epidermal cells of dark-grown PIF3/pifq (line 1-2) and PIF3mAD/pifq (line 2-1) seedlings after 1 min of exposure to 10 μmol m⁻² s⁻¹ R light. Nuclei were stained with DAPI. The scale bars represent 5 μm. The localization patterns of HA-YFP-PIF3 and HA-YFP-PIF3mAD were observed in at least five individual seedlings of each line. b Immunoblots showing the phosphorylation and degradation of HA-YFP-PIF3 and HA-YFP-PIF3mAD in 4-d-old PIF3/pifq and PIF3mAD/pifq seedlings during the dark-to-light transition at the indicated time points in 10 μmol m⁻² s⁻¹ R light. HA-YFP-PIF3 and HA-YFP-PIF3mAD were detected using anti-HA antibodies. RPN6 was used as a loading control. The immunoblot experiments were independently repeated four times, and the immunoblots of one representative experiment are shown. The numbers beneath the PIF3 immunoblot indicate the relative levels of HA-YFP-tagged PIF3 or PIF3mAD relative to the abundance of each line in the darkness. The right panel shows the kinetic changes of HA-YFP-PIF3 and HA-YFP-PIF3mAD during the dark-to-light transition using data from all four experiments. The protein levels of PIF3 and PIF3mAD were quantified relative to those at time 0. Although PIF3mAD/pifq accumulated slightly less HA-YFP-PIF3mAD than HA-YFP-PIF3 in PIF3/pifq in darkness, the degradation kinetics of HA-YFP-PIF3mAD was the same as that of HA-YFP-PIF3. Error bars represent the s.d. of four biological replicates. The centers of the error bars represent the mean values. The statistical significance was analyzed using two-tailed Student’s t-test. n.s. indicates the values between the PIF3/pifq and PIF3mAD/pifq samples are not significantly different. c qRT-PCR results showing the steady-state transcript levels of ELIP2 in seedlings of 4-d-old dark-grown Col-0, pifq, and the transgenic PIF3/pifq and PIF3mAD/pifq lines before (D) and 1 h after (R1) exposure to 10 μmol m⁻² s⁻¹ R light. The transcript levels were calculated relative to those of PP2A. The numbers represent fold changes of the transcript level of ELIP2 in R1 compared to that in D. Error bars represent the s.d. of three biological replicates. The centers of the error bars represent the mean values. The statistical significance was analyzed using two-tailed Student’s t-test (\( **P \leq 0.01, ***P \leq 0.001, ****P \leq 0.0001 \)). The source data underlying the immunoblots in (b) and qRT-PCR data in (c) are provided in the Source data file.
Interestingly, coexpressing PHYA in yeast also inhibited the transactivation activity of PIF3 and PIF1 in a light-dependent manner (Fig. 7e), suggesting binding of PHYA to APA can also inhibit the AD activity.

**Fig. 6 PIF3’s AD participates in gene activation by shade.** a Images of representative 4-d-old seedlings of Col-0, pifq, two PIF3/pifq lines, and two PIF3mAD/pifq lines grown in 10 μmol m⁻² s⁻¹ R light. b Hypocotyl length measurements of the lines shown in (a). The boxes represent the 25th to 75th percentiles; the bars are equal to the median values; the whiskers extend to the maximum and minimum data points. Samples labeled with different letters exhibited statistically significant differences in hypocotyl length (ANOVA, Tukey’s HSD, *P* ≤ 0.05). c Immunoblot showing the steady-state levels of HA-YFP-PIF3 or HA-YFP-PIF3mAD in the PIF3/pifq and PIF3mAD/pifq seedlings shown in (a) using anti-HA antibodies. RPN6 was used as a loading control. The immunoblot experiments were independently repeated at least three times, and the results of one representative experiment are shown. The numbers beneath the immunoblot indicate the relative levels of HA-YFP-PIF3 or HA-YFP-PIF3mAD normalized to RPN6. d qRT-PCR results showing the steady-state transcript levels of select PIF3 target genes in 4-d-old R-light-grown seedlings of Col-0, pifq, and the PIF3/pifq and PIF3mAD/pifq lines before (R) and after a 1-h FR treatment (FR). The transcript levels were calculated relative to those of PP2A. Error bars represent the s.d. of three biological replicates. The centers of the error bars represent the mean values. Numbers indicate fold changes relative to pifq; the statistical significance was analyzed using two-tailed Student’s *t*-test (*P* ≤ 0.05, **P* ≤ 0.01, ***P* ≤ 0.001, ****P* ≤ 0.0001); n.s. indicates the difference was either <2-fold or not statistically significant. The source data underlying the immunoblots in (c) and qRT-PCR data in (d) are provided in the Source data file.

**Fig. 7 NGB represses PIF3 activity via the light-sensing knot.** Previous studies have identified three mutations in PHYB’s light-sensing knot, R110Q, G111D, and R352K, which disrupt the PHYB-APB interaction. We examined whether these mutations could attenuate the effect of NGB on PIF3’s activity. Indeed, all three mutations in the light-sensing knot significantly reduced the inhibitory function of NGB on PIF3’s AD activity (Fig. 8a). NGB can rescue the long hypocotyl phenotype of the null phyB-5 mutant. The in vivo activity of NGB relies on its interaction with the APB motif in PIFs, as NGB-R110Q, NGB-G111D, and NGB-R352K failed to rescue phyB-5. Because NGB has little activity in promoting PIF3 degradation, it was proposed that NGB regulates hypocotyl growth by inhibiting the function of PIF3. Our new findings raised the possibility that NGB controls hypocotyl growth by directly repressing the AD activity of PIF3. To test this hypothesis, we grew NGB, NGB-R110Q, NGB-G111D, and NGB-R352K seedlings in continuous R light and compared the protein levels of PIF3 as well as the transcript levels of select PIF3 target genes. PIF3 accumulated to similar levels in NGB, NGB-R110Q, NGB-G111D, NGB-R352K, and phyB-5, confirming that NGB, as well as the NGB mutants, have little activity in promoting PIF3 degradation in the light (Fig. 8b). However, despite the similar levels of PIF3, the transcript levels of PIF3 target genes in NGB remained low and were similar to those in *pbg*, in which PIF3 was undetectable (Fig. 8b, c), suggesting that NGB can almost fully block the activity of PIF3 in vivo. The transcriptional repression activity of NGB was greatly compromised in NGB-R110Q, NGB-G111D, and NGB-R352K, as all three mutants showed elevated transcript levels of PIF3 target genes similar to those in phyB-5 (Fig. 8c), indicating that disrupting the PHYB-APB interaction almost completely abolished the transcriptional repression activity of PIF3.
NGB. These results thus provide genetic evidence supporting the model that binding of PHYB’s light-sensing knot to the APB motif of PIF3 directly represses the transactivation activity of the PIF3 AD in vivo.

Discussion

The PHYB-PIF signaling module is at the center of plant-environment interactions that controls the expression of hundreds of light-responsive genes, thereby profoundly influencing almost all aspects of plant development, growth, metabolism, and immunity. However, the AD sequences of PIFs and the regulatory mechanism of PIFs’ intrinsic AD activity had not been precisely determined. This study defines a 24-amino-acid AD in PIF3 and identifies the functional attributes of the PIF3 AD including an essential ΦxxΦΦ activator motif and flanking acidic residues, which strongly resemble the ADs of the mammalian tumor suppressor p53 and the yeast activator Gcn4. These findings reveal the unexpected conservation of sequence-specific ADs across the animal, fungal, and plant kingdoms.

Moreover, we uncovered a novel PHYB signaling mechanism via direct photoinhibition of the AD activity of PIF3 (Fig. 9). These results, combined with those of previously published studies on the structure–function relationship of PHYB, indicate that the transactivation activity and stability of PIF3 are controlled by structurally separable mechanisms via PHYB’s N-terminal photosensory and C-terminal output modules, respectively (Fig. 9). While the light-independent interaction between the C-terminal module of PHYB and PIF3 promotes PIF3 degradation, the light-reversible interaction between the N-terminal photosensory module of PHYB and the APB of PIF3 directly modulates PIF3’s transactivation activity (Fig. 9). We propose that PHYB, and likely PHYA, regulates the stability and activity of other PIFs also via the dual mechanisms.

We demonstrated that PIF3 possesses a single AD in the aa91-114 region between the APB and APA motifs. The PIF3 AD defined by this study is consistent with the previously identified activating region in yeast between amino acids 90 and 120. We further narrowed down the AD region and, more importantly,
Fig. 8 NGB inhibits PIF3 activity via light-sensing knot. a Results of yeast liquid β-galactosidase assays showing the activity of PIF3 expressed alone (-) or coexpressed with NGB or NGB mutants. The transactivation activities were calculated relative to that of the yeast strains expressing PIF3 alone. Samples labeled with different letters exhibited statistically significant differences in transactivation activity (ANOVA, Tukey’s HSD, P < 0.05, n = 3). The numbers represent fold-changes between the NGB mutants and NGB. Error bars represent the s.d. of three biological replicates. The centers of the error bars represent the mean values. The source data underlying the yeast liquid immunoblots in (b) are shown. Representative experiment are shown. b Immunoblots showing the steady-state levels of PBG, NGB, and PIF3 in 4-d-old Ler, phyB-5, PBG, NGB, NGB-R110Q, NGB-G111D, and NGB-R352K seedlings grown in 10 μmol m⁻² s⁻¹ R light. PBG and NGB were detected using anti-GFP antibodies, and PIF3 was detected using anti-PIF3 antibodies. RPN6 was used as a loading control. The immunoblot experiments were independently repeated at least three times, and the results of one representative experiment are shown. c qRT-PCR results showing the steady-state transcript levels of PIF3 target genes, PIL1, XTR7, ATHB2, and IAA29, in the lines described in (b). The transcript levels were calculated relative to those of PPA2A. The numbers indicate fold changes relative to NGB; the statistical significance was analyzed using two-tailed Student’s t-test (**P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001). Error bars represent the s.d. of three biological replicates. The centers of the error bars represent the mean values. The source data underlying the yeast liquid β-galactosidase assays in (a), the immunoblots in (b), and the qRT-PCR data in (c) are provided in the Source data file.

showed that this is the only AD in PIF3, revealed its similarities to the ADs of p53 and Gcn4, validated the function of the PIF3 AD in vivo, and uncovered the direct regulation of the PIF3 AD by PHYB. The conclusion that PIF3 possesses a single p53-like AD is supported by both transactivation assays in yeast and the characterization of the PIF3mAD mutant in planta. First, truncation analysis and alanine-scanning mutagenesis of PIF3 demonstrated that aa91-114 is the only region necessary and sufficient for PIF3’s AD activity in yeast (Fig. 1 and Supplementary Fig. 1). Different from the prior work, which suggested a second AD between amino acids 27 and 43, aa27-43 did not show any AD activity in our experiments (Fig. 1 and Supplementary Fig. 1). Second, the PIF3 AD shares similar attributes as the ADs of p53 and Gcn4, including an ΦxxΦΦ activator motif and flanking acidic residues (Fig. 2a). Third, the PIF3mAD mutant in which the ΦxxΦΦ activator motif is replaced with five alanines compromises the transactivation functions of PIF3 in both yeast and Arabidopsis (Figs. 2, 4, 5, and 6), providing strong evidence validating the function of the PIF3 AD. The PIF3 AD is highly conserved in PIF3 orthologs in eudicots, suggesting evolutionarily-conserved roles in PIF3’s activator function (Fig. 2a).

Our results unveil a novel mechanism of PHYB signaling via direct photoinhibition of the transactivation activity of PIF3. The PIF3 AD resides between the APB and APA motifs (Fig. 9)24,66,67. We showed that the light-induced interaction between the N-terminal photosensory module of PHYB and the APB repressed the transactivation activity of PIF3 in yeast, whereas the C-terminal output module of PHYB had no effect on PIF3’s activity (Fig. 7b, c). Consistent with the conclusion, the inhibitory effect of PHYB on PIF3’s activity was significantly attenuated when the PHYB-APB interaction was disrupted by the R110Q, G111D, and R352K mutations in the light-sensing knot of PHYB (Fig. 8a)66,67. Furthermore, Using NGB, which contains only the N-terminal photosensory module of PHYB and the APB motif of PIF3 is required for repressing the transactivation activity of PIF3 in vivo (Fig. 8b, c)17,41. These results thus uncover a new signaling mechanism by the photosensory module of PHYB via direct inhibition of PIF3’s AD activity, possibly by blocking the AD from binding to transcriptional coactivators. Our results confirmed that NGB or the N-terminal module of PHYB is not responsible for promoting PIF3 degradation
The sequestration activity of PHYB relies on target gene promoters independently of PHYB-mediated PIF5, and PIF7 gene regulation, and also, it is likely a critical mechanism to transactivation activity provides a rapid mode of photoresponsive signaling. PHYA could inhibit the activity of PIF3 and PIF1 in yeast (Fig. 7e), in parallel, the C-terminal output module, which binds PHYA to APB also inhibits the activity of the PIF3 AD. The schematic of PHYB domain structure is modified from a model by Burgie and Viestra.

Consistent with this conclusion, it was shown previously that a constitutively active version of the photosensory module of PHYB, which harbors the Y276H mutation, also failed to trigger PIF3 degradation in the light. Together, these results, combined with previously published data on the function ofPHYB’s C-terminal output module in PIF3 degradation, indicate that PHYB regulates the activity and stability of PIF3 through structurally separable actions of the N-terminal photosensory module and C-terminal output module, respectively (Fig. 9).

Because the PIF3 AD is conserved in other PIF members including PIF1, PIF4, PIF5, PIF7, and PIF8 (Fig. 2b, c), it is likely that PHYB also controls these PIFs via the dual mechanisms. Supporting this notion, the photosensory module of PHYB can inhibit the activity of PIF1 in a light-dependent manner in yeast (Fig. 7d). Moreover, our results showed that photoactivated PHYA could inhibit the activity of PIF3 in yeast (Fig. 7e), suggesting that PHYA may also transduce light signals through direct inhibition of the AD activity of PIF3 and PIF1. Compared with the mechanism of PIF degradation, direct inhibition of PIFs’ transactivation activity provides a rapid mode of photoresponsive gene regulation, and also, it is likely a critical mechanism to regulate the PIFs that can accumulate in the light, such as PIF4, PIF5, and PIF7.

PHYS triggers rapid phosphorylation of PIF3 by photo-regulatory protein kinases (PPKs) in the light. The PIF3 AD contains two light-dependent phosphorylation sites, S102 and S108. Phosphorylation at these sites is expected to increase negative charges around the activator motif, which could potentially alter the accessibility of the activator motif and/or the affinity of the AD to transcriptional coactivators. Therefore, PHYB and PHYA may also modulate the activity of the PIF3 AD through phosphorylation. This hypothesis needs to be further examined in future studies.

The interaction between PHYB and PIF3 was shown to reduce PIF3’s DNA-binding activity or enhance PIF3 sequestration away from target gene promoters independently of PHYB-mediated PIF3 degradation. The sequestration activity of PHYB relies only on PHYB’s N-terminal module and is abolished by the G111D mutation in the light-sensing knot. Our results here thus raised the question of the relationship between PIF3’s transactivation activity and DNA binding. To discern PHYB’s function in inhibiting the PIF3 AD from its role in regulating PIF3 DNA-binding activity, we chose to use the PIF3-N4 fragment to measure the photoinhibition of PIF3’s AD by PHYB, because PIF3-N4 does not contain the bHLH domain, and therefore, its DNA binding in yeast relies on the Gal4-DBD instead (Fig. 7a, b). These experiments demonstrate that the N-terminal module of PHYB can directly inhibit the transactivation activity of PIF3, independently of PIF3’s bHLH activity—i.e., the photoinhibition of the activity of the PIF3 AD by PHYB is not a consequence of a reduction in PIF3’s DNA-binding activity. It is conceivable that blocking the activity of PHYB’s AD by PHYB may disrupt the interactions of PIF3 to transcriptional coactivators, thereby reducing PIF3’s association to target gene promoters. Alternatively, it is equally possible that, in addition to repressing the transactivation activity, binding of PHYB to the APB motif of PIF3 also triggers conformational changes that allosterically reduce the DNA-binding activity of the bHLH domain. The current data cannot distinguish between these two models.

It was demonstrated more than 30 years ago that a transcriptional activator from one species, such as the yeast activator Gal4 and the herpes simplex virus protein VP16, can act as a potent activator in animal, yeast, and plant cells, leading to the notion that the basic mechanism of transcription activation is likely conserved in eukaryotes. However, it has been puzzling why transcription can be activated by diverse AD sequences across eukaryotic kingdoms. The identification of the sequence-specific ΦxxΦ motif in animal and yeast activators suggests that the mechanism of transcriptional activation could be conserved at the primary sequence level and the intrinsic disordered property of ADs. Our knowledge, the PIF3 AD is the first sequence-specific AD identified in plants. Our results therefore provide initial evidence supporting the conservation of sequence-specific ADs across the animal/fungal and plant kingdoms. In conclusion, our results identify a p53-like AD in the PIF family of transcriptional activators and unveil a novel light signaling mechanism via direct photoinhibition of PIFs’ transactivation activity by PHYB and likely PHYA. It will be of interest in future work to investigate the transactivation mechanism by PIFs’ ADs and how binding of PHYB inhibits such mechanism.

Methods

**Plant materials, growth conditions, and hypocotyl measurement.** *Arabidopsis* wild-type Columbia (Col-0) and Landsberg erecta (Ler), as well as the pif2 (Col-0), pif145, and phyB-5 (Ler) mutants, were used as controls to characterize hypocotyl growth and gene expression under various light conditions. The PBG, NGB, NGB-R100Q, NGB-G111D, and NGB-R352K lines have been previously reported. To generate the plasmids containing the wild-type CDS sequences of PBG, NGB, and NGB2-1, the PBGmAD/pf1q (1-2 and 9-5) and PIF3mAD/pf1q (2-1 and 4-5) transgenic lines were generated in this study.

Arabidopsis seeds were surface-sterilized and plated on half-strength Murashige and Skoog medium containing Gamborg’s vitamins (Caisson Laboratories), 0.5 mM MES pH 5.7, and 0.8% agar (w/v). Seeds were stratified in the dark at 4 °C for 5 days and grown at 21 °C in an LED chamber (Perceval Scientific, Perry, IA) in the indicated light conditions. Fluence rates of light were measured with an Apogee PS200 spectroradiometer (Apogee Instruments, Logan, UT) and SpectraWiz spectroscopy software (StellarNet, Tampa, FL). Images of representative seedlings were taken using a Leica MZ FLIII stereo microscope (Leica Microsystems Inc., Buffalo Grove, IL) and processed using Adobe Photoshop CC (Adobe Inc., Mountain View, CA). For hypocotyl measurements, seedlings were scanned with an Epson perfection V700 photo scanner, and hypocotyl length was measured using the NIH ImageJ software. Box-and-whisker plots of hypocotyl measurements were generated using the Prism 8 software (GraphPad Software, San Diego, CA).

**Plasmid construction and generation of transgenic lines.** The PCR primers used to generate the plasmids containing the wild-type CDS sequences of PIFs, PHYA, and PHYB are listed in Supplementary Table 1. All PIF3 constructs for the yeast transactivation assays were generated by subcloning either the full-length or a fragment of PIF3 CDS into the EcoRI and SacI sites of the pBR vector (TaKaRa NATURE COMMUNICATIONS | (2021) 12:5614 | https://doi.org/10.1038/s41467-021-25909-5 | www.nature.com/naturecommunications
Bio USA, San Jose, CA). The GST-PF3 and GST-PF3mAD constructs for GST pulldown assays were generated by subcloning PF3 or PF3mAD into the EcoRI and XbaI sites of the pET24b vector (Promega, Madison, WI). The vectors for examining the transactivation activity of the Pf3 paralogs were generated by full-length CD sequences of the Pf3 paralogs into the EcoRI and SalI sites of the pBrige vector using HiFi Assembly. The constructs for testing the function of PHB and PHYA in inhibiting the transactivation activity of Pf3 and Pf3 were generated by subcloning the DNA 300-500-bp coding full-length PHB or PHA fused with SV40 NLS, PHB-C (PHB amino acids 594-1172 fused with SV40 NLS), and NGB (PHB amino acids 1-651 fused with GFP, GS, and SV40 NLS) into the Nol and NdeI sites of the pBrige-PF3-N4, pBrige-PF3-M1, pBrige-PF3-PIF, or pBrige-PF1 plasmid vectors.

The constructs containing mutants of Pf3 or PHBY were generated by one of the following two methods. For Pf3 alanine scanning mutants m1 to m15, the mutants were generated in pBrige-PF3-N1 with the Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Ipswich, MA). The primers used to generate the m1 to m15 mutants are listed in Supplementary Table 2. For the rest of the Pf3 and PHBY point mutants constructs, two new primers from either the sense or antisense strands harboring the mutations were designed and used in combination with the primers flanking the 5′ and 3′ ends of the respective CD sequences (Supplementary Table 1) to amplify two overlapping fragments of the respective CD sequences. The two overlapping DNA fragments were then ligated to the indicated vectors using HiFi Assembly. The pairs of primers containing the mutated nucleotide sequences are listed in Supplementary Table 2.

Yeast transactivation assay. Cell viability assays were performed by using either 2YHGold yeast strains (Takara Bio USA, San Jose, CA) containing a pBridge bait vector or diploid yeast strains generated by mating a 2YHGold strain containing a bait vector with a Y187 strain (Takara Bio USA, San Jose, CA) containing the pGADT7 vector. Overnight yeast cultures were diluted to an OD600 of 0.2 and serially diluted from 10° to 10

RNA extraction and qRT-PCR. Total RNA was extracted from seedlings using a Quick-RNA MiniPrep kit with on-column DNase I treatment (Zymo Research, Irvine, CA). Quantitative RT-PCR was performed with FastStart Universal SYBR Green Master Mix and a LightCycler 96 Real-Time PCR System (Roche, Basel, Switzerland). Transcript levels of genes were calculated relative to the level of P2A. Genes and primer sets used for qRT-PCR are listed in Supplementary Table 3.

Chromatin immunoprecipitation. ChIP assays were performed using chromatin isolated from 4-d-old dark-grown PF3/pf3 and PF3mAD/pf3 transgenic lines. Seedlings were ground in liquid nitrogen and resuspended in nuclear isolation buffer (10 mM HEPES pH 7.6, 1 mM MgCl2, 1 mM KCl, 5 mM EDTA, 14 mM β-mercaptoethanol, protease inhibitor cocktail, 40 μg/mL PMSF, 5 mM EDTA, and 50 μg/mL MG115) containing 1% formaldehyde, 0.6% Triton X-100, and 0.4 mM PMSF. Samples were incubated at room temperature for 10 min for cross-linking, and then 125 μl glycine was added to terminate the cross-linking. The lystate was incubated at 3000 x g for 10 min. The nuclear pellet was resuspended in nuclear isolation buffer, loaded on top of a 15% Percoll solution (15% Percoll, 10 mM HEPES pH 8.0, 1 M sucrose, 5 mM KCl, 5 mM MgCl2, 5 mM EDTA), and centrifuged at 3000 x g for 5 min. The enriched nuclear pellet was lysed with nuclease lysis buffer (50 mM Tris-HCl pH 7.5, 1% SDS, 10 mM EDTA). Then, sonication was performed using a Covaris S2 ultrasonicator (Covaris, Inc., Woburn, MA). The lysate was sonicated at 13,000 x g for 3 min at 4 °C to remove any debris. The nuclear lysate was diluted with ChIP dilution buffer (15 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100). The lysate was incubated with 1 μg of rabbit polyclonal anti-HA-AN (Abcam, cat. no. ab9110) or rabbit IgG (Cell Signaling Technology, cat. no. 27295) for 3 h at 4 °C. Immunoprecipitated chromatin with anti-HA antibody or IgG was then incubated with Dynabeads Protein G (ThermoFisher Scientific, Waltham, MA) for 2 h. The beads were washed with 2 mM NaHCO3. Reverse cross-linking was performed by adding 20 μl of 5 M NaCl to the eluates and incubating at 65 °C overnight. Then, the immunoprecipitated proteins were digested by adding 10 μl of 0.5 M EDTA pH 8.0, 20 μl of 1 M Tris-HCl pH 6.5 and 20 μg Proteinase K and incubating at 50 °C (5 μl). The final chroma was purified with ChIP DNA Clean & Concentrator (Zymo Research, Irvine, CA). Primer sets used for ChIP-qPCR are listed in Supplementary Table 4.

Protein extraction and immunoblots. Total proteins from 100 mg of 4-d-old seedlings were extracted in 300 μl of extraction buffer (100 mM Tris-HCl pH 7.5, 100 mM NaCl, 5% glycerol, 0.5% Triton X-100, 50 mM β-mercaptoethanol, 2 mM PMSF, cOmplete™ Protease Inhibitor Cocktail, 80 mM MgCl2 and 80 mM MgCl115, 1% phosphatase inhibitor cocktail 3, 10 mM N-ethylmaleimide, and 0.01% bromophenol blue). Samples were immediately boiled for 10 min and centrifuged at 16,000 x g for 10 min. Proteins were separated via SDS-PAGE and blotted onto a nitrocellulose membrane. The membrane was first probed with the indicated primary antibodies and then incubated with goat anti-rabbit (Bio-Rad, cat. no. 1706513) secondary antibodies conjugated with horseradish peroxidase at a 1:5000 dilution. The signals were detected via a chemiluminescence reaction using the SuperSignal West Dura Extended Duration Substrate (ThermoFisher Scientific, Waltham, MA). Rabbit polyclonal anti-PF3, goat polyclonal anti-AN (GenScript, cat. no. A00168), rabbit polyclonal anti-GFP (Abcam, cat. no. ab290), and rabbit polyclonal anti-RPN6 (Enzo Life Sciences, cat. no. BML-PW8370-0100) antibodies were used at a 1:1000 dilution.

Confocal imaging. Confocal analysis of HA-YFP-PF3 and HA-YFP-PF3mAD was performed as previously described with minor modifications56. Seedlings were fixed in 2% paraformaldehyde in PBS under vacuum for 15 min and then iced with 300 mM NHexi in PBS for 5 min three times. The seedlings were permeabilized with 0.02% Triton X-100 in PBS for 5 min, incubated with 300 nM DAPI in PBS for 10 min. The seedlings were washed three times with PBS for 5 min three times, and mounted with ProLong™ Diamond Antifade (ThermoFisher Scientific, Waltham, MA) on a slide. The slides were left to cure overnight in the dark, sealed with nail polish, and stored at 4 °C until imaging. Nuclear of hypocotyl cells of normal cells were imaged using a Zeiss 108 inversion microscope equipped with a Plan-Apochromat 100×1.4 oil-immersion objective and an Axioscan 506 mono camera (Carl Zeiss, Jena, Germany). Fluorescence was detected with the following Zeiss filter sets: YFP, exciter 300/25 nm/nm, emitter 535/40 nm/nm (Zeiss Filter Set 46); DAPI, exciter 365 nm, emitter 445/50 nm/nm (Zeiss Filter Set 4). Images were collected using Zeiss ZEN software and processed using Adobe Photoshop Creative Cloud.
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Acknowledgements

We thank Dr. Peter Quail for providing the pifq mutant. We thank Dr. Elise Pasoreck for providing valuable comments and suggestions on the manuscript. This work was supported by National Institute of General Medical Sciences Grants R01GM087388 and R01GM132765 to M.C. and National Institute of Food and Agriculture hatch projects CA-R-BPS-5186-H and CA-R-BPS-5084-H to M.C. and X.C., respectively. Q.S. was supported by Guangdong Innovation Team Project 2014ZT05S078.

Author contributions

C.Y.Y., J.H., Q.S., Y.Q., L.L., B.M., X.C., and M.C. conceived the original research plan; M.C. and X.C. supervised the experiments; C.Y.Y., J.H., Q.S., Y.Q., L.L., R.J.K., E.G.C., and J.H. performed the experiments; C.Y.Y., J.H., Q.S., Y.Q., L.L., R.J.K., E.G.C., J.H., N.M., P.Z., L.C.S., A.N., B.M., X.C., and M.C. analyzed the data; A.N. provided the transgenic lines expressing NGB and NGB mutants; M.C., C.Y.Y., J.H., and Q.S. wrote the article with contributions from all authors.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-25909-5.

Peers view information Nature Communications thanks the anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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