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Phytochrome B inhibits binding of Phytochrome-Interacting Factors to their target promoters

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Summary

Phytochromes are red and far-red light receptors in plants that mediate critical responses to light throughout the life cycle. They achieve this in part by targeting negatively acting bHLH transcription factors called phytochrome-interacting factors (PIFs) for degradation within the nucleus. It is not known, however, if protein degradation is the primary mechanism by which phytochromes inhibit these repressors of photomorphogenesis. Here, we use ChIP analysis to show that phyB inhibits the regulatory activity of PIF1 and PIF3 by releasing them from their DNA targets. The N-terminal fragment of phyB (NG-GUS-NLS; NGB) also inhibits the binding of PIF3 to its target promoters. Unlike the full-length phyB, however, NGB does not promote PIF3 degradation, establishing the activity of NGB reflects its ability to inhibit PIFs' binding to DNA. We further show that Pfr forms of both full-length phyB and NGB inhibit the DNA binding of PIF1 and PIF3 in vitro. Taken together, our results indicate that phyB inhibition of PIF function involves two separate processes, sequestration and protein degradation.

Keywords

Phytochrome; PIF3; PIF1; Degradation; Phosphorylation; DNA binding

Introduction

Phytochromes are red and far-red light photoreceptors that modulate key physiological and developmental processes of plants including seed germination, seedling photomorphogenesis, shade avoidance, and flowering (Rockwell *et al.* 2006). At least two branches of phytochromes have evolved to facilitate adaptation to the variation in the light spectrum found in common terrestrial environments (Franklin and Quail 2009, Mathews 2006). One branch, typified by Arabidopsis phytochrome B (phyB), mediate the so-called low fluence responses (LFRs), i.e. those strongly activated by red light common in open field plantings. The other branch, typified by Arabidopsis phytochrome A (phyA), mediate the very low fluence response (VLFR) and far-red high irradiance response (FR-HIR) – processes both activated by the far-red light enriched environments of deep canopy shade and in dense crop plantings. While the distinct response modes of phyA and phyB categories appear well conserved in flowering plants, phyA can also function as a LFR sensor depending on the plant species and stage of development (Franklin *et al.* 2007, Takano *et al.*

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2005). In spite of this photosensory diversity, the regulatory gene networks targeted by all classes of plant phytochromes appear to significantly overlap and share common regulatory factors.

Phytochrome interacting proteins play a key role in assisting all classes of phytochromes to regulate light responses. Some interacting proteins regulate specific phytochrome activity by modulating their subcellular localization, their stability, or their light-independent thermal reversion (dark reversion) to inactive species, whereas others regulate light responses directly under phytochrome control (Bae and Choi 2008). The most well-studied of these are the so-called phytochrome-interacting bHLH transcription factors, termed PIFs or PILs (PIF3-like proteins), which primarily function as negative regulators of photomorphogenesis singly and/or in combination (Leivar *et al.* 2008, Leivar and Quail 2011, Shin *et al.* 2009). Seed germination is one example of a light response that is mainly regulated by a single PIF (Oh *et al.* 2004). By contrast, light-dependent chloroplast development, inhibition of hypocotyl elongation, and negative hypocotyl gravitropism are redundantly regulated by more than one PIF (Fujimori *et al.* 2004, Huq *et al.* 2004, Huq and Quail 2002, Kim *et al.* 2003, Lorrain *et al.* 2008, Lorrain *et al.* 2009, Nozue *et al.* 2007, Oh, *et al.* 2004, Shin, *et al.* 2009, Stephenson *et al.* 2009). Consistent with the redundant negative roles of PIFs, *pif1pif3pif4pif5* quadruple mutants display constitutive photomorphogenic phenotypes (Leivar, *et al.* 2008, Shin, *et al.* 2009), expressing light inducible genes even in the dark (Leivar *et al.* 2009, Shin, *et al.* 2009). It has thus been argued that photomorphogenesis is the consequence of targeted removal of PIFs that regulate skotomorphogenetic development, i.e. the heterotrophic genetic pathway(s) that sustains seed and seedling viability/development on stored food reserves in the absence of light – a critical hallmark of seed plant development (Leivar and Quail 2011).

It is well accepted that phytochromes promote light responses, in part, by destabilizing PIFs at the protein level. When phytochromes are activated by light, they enter the nucleus where they bind to PIFs and target them for degradation (Bauer *et al.* 2004, Khanna *et al.* 2004, Kircher *et al.* 1999, Lorrain, *et al.* 2008, Ni *et al.* 1998, Nozue, *et al.* 2007, Oh *et al.* 2006, Park *et al.* 2004, Sakamoto and Nagatani 1996, Shen *et al.* 2005, Yamaguchi *et al.* 1999). Binding analysis has shown that different phytochromes bind to PIFs through different motifs. Phytochrome B (phyB) interacts with an APB motif that can be recognized in 12 bHLH proteins, including four negatively acting PIFs (Khanna, *et al.* 2004). By contrast, phytochrome A (phyA) interacts with unrelated and distinct APA motifs found in PIF1 and PIF3 (Al-Sady *et al.* 2006, Shen *et al.* 2008). Despite different binding motifs, both phyA and phyB induce rapid phosphorylation of the PIFs to which they interact and their subsequent degradation via the 26S proteasome (Al-Sady, *et al.* 2006, Lorrain, *et al.* 2008, Nozue, *et al.* 2007, Oh, *et al.* 2006, Park, *et al.* 2004, Shen, *et al.* 2005). Since mutations in APA and APB that prevent this light-dependent interaction inhibit both phosphorylation and degradation of PIFs, the hypothesis that phosphorylation primes the degradation of PIFs has been proposed (Al-Sady, *et al.* 2006). While the nature of the kinase(s) that mediate PIF phosphorylation has not yet been resolved, plant phytochromes possess regulatory domains that bear striking resemblance to two component histidine kinase sensors of prokaryotes (Schneider-Poetsch *et al.* 1991) and evidence that some plant phytochromes can function as serine-threonine protein kinases provides an argument that phytochrome itself may play a role in this process (Yeh and Lagarias 1998).

Analysis of transgenic plants expressing truncated phytochromes suggested that N-terminal photosensory domains themselves are functional (Matsushita *et al.*, 2003; Oka *et al.*, 2004; Mateos *et al.*, 2006) and mutated/truncated alleles of phytochromes that lack the ATP-binding motif in the C-terminal region retain photoregulatory activity (Krall and Reed 2000). Further detailed analyses of the structural basis of PIF phytochrome interaction

support the conclusion that N-terminal photosensory ‘input’ domains of plant phytochromes are wholly sufficient to sustain the light-dependent interaction with PIFs (Kikis *et al.* 2009, Oka *et al.* 2008, Shimizu-Sato *et al.* 2002). However, PIFs also bind to the C-terminal region of phytochromes and the presence of loss-of-function point mutations in this region made it difficult to exclude the signaling role of the C-terminal region (Ni, *et al.* 1998, Rockwell, *et al.* 2006). It is therefore conceivable that these severely truncated hyperactive alleles either misregulate PIF function in a light-dependent manner via a gain-of-function sequestration mechanism among other inhibitory mechanisms.

The present work was undertaken to address this sequestering mechanism. First, we addressed the question whether PIF3 is destabilized by its interaction with the N-terminal domain of phyB (NG-GUS-NLS; NGB). These studies indicate that this interaction does not support PIF3 degradation. Second we addressed whether DNA binding by PIFs is inhibited by phyB without being degraded. ChIP analyses show that the binding of PIF1 or PIF3 to its target promoter is inhibited both by NGB and by full length phyB. In vitro binding assays further show that Pfr forms of full-length and N-terminal truncated phyB inhibit the binding of PIFs to their target DNA. Thus, our results suggest that phyB inhibits PIFs in two ways: by releasing them from target promoters and by accelerating their turnover.

Results

Previous reports have shown that truncated phytochromes lacking their HKRD are still capable of inducing light responses (Krall and Reed 2000, Mateos *et al.* 2006, Matsushita *et al.* 2003). Whether such phytochrome N-terminal domains induce light responses via the same molecular mechanism as their full-length counterparts is not known. Degradation of four negatively acting phytochrome-interacting bHLH transcription factors (PIFs) is one of the salient features of phytochrome signaling. We therefore investigated whether a previously described nuclear-targeted N-terminal truncated Arabidopsis phyB (NG-GUS-NLS; NGB) (Matsushita, *et al.* 2003) can induce the degradation of PIF3. To obtain a phyB-deficient line that overexpresses both NGB and PIF3, the *NGB/phyB* line expressing the N-terminal 651 amino acid photosensory domain of phyB fused to GFP, GUS and an NLS (Matsushita, *et al.* 2003), was crossed with the *PIF3-OX/phyB* and *PIF3-OX/phyA* lines expressing a recombinant myc-tagged PIF3 (Park, *et al.* 2004).

A homozygous *NGB/PIF3-OX/phyB* line was then examined to test whether NGB can induce PIF3 degradation. To minimize involvement of phyA in this process, plants were grown under continuous red light (Rc) – conditions that promote phyA degradation and strongly repress *PHYA* transcription. The level of PIF3 was strongly reduced in Rc-grown *PIF3-OX* seedlings as expected for plants containing phyB, while by contrast, PIF3 levels in Rc-grown *PIF3-OX/phyB* plants were unchanged when compared with their dark-grown counterparts (Figure 1A). These results show that phyB is the major phytochrome involved in targeted degradation of PIF3 under Rc. Unexpectedly, PIF3 levels in Rc-grown *NGB/PIF3-OX/phyB* line were the same as those found in plants grown in darkness, demonstrating that NGB does not promote PIF3 degradation. Taken together, these results indicate that the hyperactive phenotype of the *NGB* transgenics does not correlate with PIF3 turnover.

Phosphorylation of PIFs (Al-Sady, *et al.* 2006, Lorrain, *et al.* 2008, Shen, *et al.* 2008) is a hallmark of phytochrome signaling. For this reason, we sought to determine whether NGB could support light-induced phosphorylation of PIF3. To minimize the role of phyA, we generated *NGB/PIF3-OX/phyA/phyB* by crossing *PIF3-OX/phyA* and *NGB/PIF3-OX/phyB*. When etiolated *PIF3-OX* control seedlings were transferred to red light and examined for PIF3 bands using Western blot analysis, slower-migrating PIF3 bands could be detected

within 20 min (Figure 1B). These slower-migrating bands were not seen in samples treated with a protein phosphatase, indicating that they were the phosphorylated forms of PIF3. Similar phosphorylated PIF3 bands were not observed in red-light-treated *PIF3-OX/phyA/phyB*, indicating that double mutations in *PHYA* and *PHYB* abolished the phosphorylation of PIF3. When NGB was introduced, the phosphorylated PIF3 bands could be detected. These results indicate that NGB restores phosphorylation of PIF3.

However, comparative analysis of the pattern of PIF3 phosphorylation revealed that full length phytochrome mediates multiple phosphorylation of PIF3 than NGB (Figure 1B: compare *PIF3-OX* and *NGB/PIF3-OX/phyA/phyB* at 20 min R). That all of these more slowly migrating species reflect phosphorylation events was ascertained by phosphatase treatment. These results indicate that NGB can mediate light-dependent phosphorylation of PIF3, but multiple phosphorylation of PIF3 requires full-length phyB. Since NGB does not activate the degradation of PIF3, our findings further correlate the multiple modification of PIFs with their turnover.

Previous studies have established that the N-terminal domain of phyB can interact with PIF3 in a light-dependent manner (Kikis, *et al.* 2009, Shimizu-Sato, *et al.* 2002). We hypothesize that such interaction may be responsible for the strong signaling activity of NGB in vivo. To test this hypothesis, co-IP experiments were performed using the *NGB/PIF3-OX* line. As shown in Figure 2A (left), NGB could be co-precipitated with PIF3 in extracts from white light grown tissue. This interaction was also observed in immunoprecipitates from R-treated extracts but not from FR-treated extracts (Figure 2A, right). These results are consistent with complex formation between PIF3 and the Pfr form of NGB in vivo.

Previous studies have established that the formation of PIF complexes with DELLA proteins, HFR1, and PAR1 and 2 have shown to strongly inhibit PIF binding to target promoters (de Lucas *et al.* 2008, Feng *et al.* 2008, Hao *et al.* 2012, Hornitschek *et al.* 2009). We therefore addressed the hypothesis that light-dependent PIF3:NGB complex formation inhibits PIF3 binding to DNA using a chromatin immunoprecipitation (ChIP) assay. Employing promoter fragments for *RGA* and *PIL1* genes as PIF target sequences (de Lucas, *et al.* 2008, Hornitschek, *et al.* 2009, Oh *et al.* 2007), we performed comparative PIF3 ChIP analyses on dark- and Rc-grown *NGB/PIF3-OX/phyB* lines. ChIPs from dark-grown seedlings proved highly enriched in both *RGA* and *PIL1* promoter target fragments compared to an *rDNA* control (Figure 2B). *RGA* and *PIL1* promoter fragments were less abundant in immunoprecipitates from Rc-grown seedlings, supporting the conclusion that PIF3 binding to both promoters is inhibited by Rc. Western blot analyses indicate that this difference was not due to higher levels of precipitated PIF3 protein in ChIPs from dark-grown seedlings (Figure 2C). We next examined the expression of *PIL1*, a gene known to be upregulated by PIFs. As expected, expression of *PIL1* was strongly repressed by red light – a response that was strongly dependent on functional phyB (Figure 2D). Red light also strongly decreased the expression of *PIL1* both in the *NGB/phyB* and *NGB/PIF3-OX/phyB* lines (Figure 2D). Taken together, our results indicate that, unlike WT phyB, NGB inhibits PIF3 in the light without triggering its degradation. NGB could inhibit PIF3 DNA binding directly by binding to PIF3 or by modifying PIF3. Alternatively, since phytochrome is known to inhibit GA biosynthesis in seedling stage (Kamiya and Garcia-Martinez 1999), NGB could inhibit PIF3 indirectly by increasing DELLA level.

These observations impelled us to test whether full-length phyB can also inhibit PIF3 binding to target promoters in a light-dependent manner. To do so, we performed ChIP analysis using dark- and Rc-grown *PIF3-OX* plant lines. Both PIF3 target promoters were greatly enriched in ChIPs from dark-grown seedlings, but not from Rc-grown seedlings (Figure 3A). To test whether PIF3 degradation in Rc was solely responsible for the result, we

also performed ChIP experiments to compare promoter occupancy in Dc- and Rc-grown *PIF3-OX* seedlings treated with the 26S proteasome inhibitor MG132. For Dc-seedlings, PIF3 was strongly associated with target promoters and this association further increased following MG132 treatment (Figure 3B), reflecting the increased PIF3 level in MG132-treated plants. Under Rc by contrast, despite the enhanced PIF3 accumulation by MG132 treatment, we could not detect any increased association of PIF3 to its target promoters. These data imply that PIF3 does not bind to its target promoters under Rc conditions that favor the phyB:PIF3 association.

An additional ChIP experiment was performed to test whether PIF1 binding to the *PIL1* promoter was similarly repressed by Rc in a *PIF1-OX* line. Like PIF3, MG132 treatment increased the PIF1 protein level, but failed to enhance its binding to the target promoter (Figure 3C). Pulse irradiation of Rc-grown seedlings with far-red light followed by short incubation in darkness was also examined. These experiments showed that the amount of target promoter recognized by both PIFs was significantly increased (Figure 3D). Taken together, these results indicate that PIF binding to target promoters is inhibited by red light even in the absence of protein turnover.

To ascertain whether R-dependent inhibition of PIF3 binding to its target promoter was phyB dependent, we performed the ChIP analysis using *PIF3-OX/phyB* mutant line. Unlike *NGB/PIF3-OX/phyB* plants, the enrichment of the PIF3 target promoter was not reduced by R-treatment in the *PIF3-OX/phyB* mutant plants (Figure 4A). Based on these results, phyB is responsible for the R-dependent inhibition of PIF3 binding to its target promoter.

Finally, to establish whether phytochrome interaction with PIFs is required to inhibit DNA binding, we generated transgenic *PIF3ΔN-OX* lines expressing myc-tagged PIF3 lacking N-terminal 300 amino acids. This PIF3 construct lacked both phy-interacting motifs (APA, APB) and its transcription activation domain, but retained the C-terminal 224 amino acids harboring DNA binding bHLH domain (Al-Sady, *et al.* 2006, Khanna, *et al.* 2004, Shen, *et al.* 2008). Due to the deletion of transcription activation domain, transgenic lines expressing PIF3ΔN displayed dominant-negative constitutive photomorphogenic phenotypes which included short hypocotyls in the dark, decreased greening of etiolated seedlings when transferred to light, and disrupted hypocotyl negative gravitropism in the dark (Figure S1). Similar constitutive photomorphogenic phenotypes were reported for an N-terminal deletion of PIF1 (Shen, *et al.* 2008). PIF3ΔN also was not degraded by light (Figure S1), which is also consistent with the essential role of phy-interacting motifs for the light-dependent degradation. ChIP analysis showed that while PIF3ΔN binds well to its target promoter, this binding is not inhibited by red light (Figure 4B). This confirms that the Pfr form of phytochrome must interact with PIF3 to inhibit its binding to target promoters.

To further investigate whether phyB can directly inhibit the DNA binding of PIFs, we performed in vitro inhibition assays using recombinant phyB, PIFs, and a biotinylated PIL1 target promoter fragment. For the assay, we first incubated biotinylated target DNA with PIFs and PIFs-bound target DNAs were separated by streptavidin resin to remove unbound PIFs. The PIF-target DNA complex was then incubated with either Pr or Pfr forms of recombinant phyB. After thorough washing, DNA-bound PIFs were eluted and were quantified immunochemically. These experiments showed that smaller amounts of PIF1 and PIF3 proteins were remained bound to target DNAs when incubated with Pfr form of phyB than with Pr form (Figure 5). We also performed the same experiment using the N-terminal domain of phyB (amino acids 1-650), which was previously shown to be functional even in the absence of the dimerization domain (Matsushita, *et al.* 2003). Similar to our findings with full-length phyB, the Pfr form of the N-terminal domain of phyB also dissociated PIF1 from the target promoter fragment (Figure 5). Taken together, our results indicate that the

Pfr forms of both full-length and N-terminal-domain phyB can directly inhibit the binding of PIFs to their target promoters.

DISCUSSION

In this study, we show that the activity of N-terminal domain of phyB (NG-GUS-NLS; NGB) (Matsushita, *et al.* 2003) is not due to targeted degradation of PIF3 in response to red light. Instead, the N-terminal phyB inhibits the binding of PIF3 to its target promoters in a red light-dependent manner in vivo. Moreover, we also show that the full length phyB also inhibits the binding of PIFs to their target promoters under red light in vivo. Indeed, Pfr forms of both full-length and N-terminal domain of phyB are capable of inhibiting the DNA binding of PIFs in vitro. Based on these observations, we propose that phyB inhibits negatively acting PIFs by two different modes of action: by releasing them from their target promoters and by mediating their degradation.

Our results also indicate that the NGB, although functionally similar to full-length phyB (Matsushita, *et al.* 2003), lacks the full regulatory activity of the full length phyB. Indeed, while both N-terminal and full-length phyB can inhibit the DNA binding of PIFs both in vivo and in vitro, NGB fails to target PIFs for degradation. In addition, when etiolated seedlings were transferred to red light, NGB yielded only one slower-migrating phosphorylated PIF3 band. By contrast, the full-length phyB afforded multiple phosphorylated bands under the same conditions. This suggests that the light-dependent interaction between the NGB and PIFs is sufficient to trigger partial PIF phosphorylation. We therefore conclude that the C-terminal domain, although dispensable for the induction of light responses (Krall and Reed 2000, Matsushita, *et al.* 2003), is required for multiple light-dependent phosphorylation of PIF3. Since NGB does not mediate PIF3 degradation under red light, we also conclude that the C-terminal domain of phyB is necessary for targeted degradation of PIFs. We hypothesize that the C-terminal domain of phyB promotes PIF turnover by mediating multiple phosphotransfers to PIFs and/or by recruiting ubiquitin E3 ligases. While phosphotransfer could be accomplished by phytochrome itself (Yeh and Lagarias 1998), the C-terminal domain of phytochrome could be responsible for recruiting a protein kinase and/or a ubiquitin E3 ligase for these functions. A previous report showed that CKII could phosphorylate PIF1 in vitro and multiple mutations in CKII target residues (Ser/Thr residues including three serine residues at its C-terminus (S464, S465, S466)) partially inhibited the light-induced degradation of PIF1 (Bu *et al.* 2011). Though such mutations still did not completely abolish the light-induced phosphorylation of PIF1, it would be interesting if CKII would play any role in multiple phosphorylation of PIFs in vivo.

Consistent with our data, previous EMSA-based studies showed that Pfr fails to form ternary complexes with the G-box element and PIF1 or PIF4 (Huq, *et al.* 2004, Huq and Quail 2002). By contrast, these reports also showed that the DNA binding activities of PIF1 and PIF4 were not disrupted by the presence of Pfr. Our results also contrast with the reported formation of a ternary complex between the Pfr form of phyB, PIF3, and the G-box-containing oligonucleotide in EMSA assay (Huq, *et al.* 2004, Huq and Quail 2002, Martinez-Garcia *et al.* 2000). While it is presently unclear what is responsible for the difference between our results and previously reported results. However, we suspect these differences may reflect the distinct protocols used. In this regard, the previous studies co-incubated phyB and PIFs in crude TnT reaction mixtures while we removed excess PIFs not bound to DNA prior to addition of phyB. When all three components (PIF1, phyB, and the target DNA fragment) were incubated together without removing excess PIF1 prior to phyB addition, similar levels of PIF1 co-precipitated with the DNA fragment irrespective of Pr or

Pfr in our hands. This suggests that free PIF1 should be removed to observe the effect of Pr and Pfr.

DNA binding by transcription factors is extensively regulated. Multiple modes of regulation have been reported for various transcription factors in different organisms. For example, DNA binding of a transcription factor can be regulated by controlling its nuclear localization. Among plant transcription factors, REPRESSION OF SHOOT GROWTH (RSG), a basic leucine zipper transcription factor regulating GA biosynthesis, is actively exported out from the nucleus and is held by its binding protein 14-3-3 in the cytosol in its inactive state (Igarashi *et al.* 2001). Similarly, BRASSINAZOLE RESISTANT1 (BZR1) and BRI1 EMS SUPPRESSOR1 (BES1), two positive brassinosteroid (BR) signaling transcription factors, are also actively exported out from the nucleus upon BR treatment (Bai *et al.* 2007, Gampala *et al.* 2007, Ryu *et al.* 2007, Ryu *et al.* 2008). DNA binding of a transcription factor can be also regulated by controlling its ability to bind DNA. This can be accomplished by physical association with other proteins, e.g. by disruption of DNA binding of the class III homeodomain-leucine zipper (HD-ZIP III) by interaction with LITTLE ZIPPER (ZPR) protein (Wenkel *et al.* 2007), or by covalent modification such as phosphorylation, which has been shown to inhibit DNA binding for many transcription factors, i.e. ELONGATED HYPOCOTYL5 (HY5), BZR1, BZR2, AUXIN RESPONSE FACTOR 2, and HEAT-SHOCK TRANSCRIPTION FACTOR1 (HSF1) (Gampala, *et al.* 2007, Hardtke *et al.* 2000, Reindl *et al.* 1997, Vert and Chory 2006, Vert *et al.* 2008).

Indeed, regulation of DNA binding by PIFs appears to be regulated by multiple factors. For example, it is well established that the DELLA repressors of GA signaling, bind to bHLH motifs of PIFs (PIF3, PIF4) and prevent PIF binding to target promoters (Cheminant *et al.* 2011, de Lucas, *et al.* 2008, Feng, *et al.* 2008). This inhibition of PIF DNA binding by DELLA proteins has been suggested to play a key role for coordinating GA signaling and light signaling. HFR1, a positive far-red light signaling component encoding an atypical bHLH protein, has been shown to heterodimerize with PIFs to inhibit their binding to target promoters (Hornitschek, *et al.* 2009). Indeed, the positive role of HFR1 in far-red enriched shade condition has been attributed to its ability to disrupt DNA binding of negatively acting PIFs. Similar to HFR1, other atypical HLH proteins, PAR1 and PAR2, can heterodimerize with PIF4 to inhibit its binding to target promoters (Hao, *et al.* 2012). Here, we show that light-activated phyB, like the DELLA proteins, HFR1, PAR1, and PAR2, can also function as a PIF-binding proteins to inhibit its DNA binding activity. Our results support a regulatory model for phytochrome signaling in which the light-dependent interaction between PIFs and phyB inhibits the binding of these bHLH transcription factors to their target promoters, effecting regulation of gene expression. It is interesting that the mode of interaction of each of the three classes appear distinct, i.e. phytochromes bind to N-terminal motifs of PIFs, DELLAs bind to the bHLH motif of PIFs (de Lucas, *et al.* 2008, Feng, *et al.* 2008), and HFR1, PAR1, and PAR2 heterodimerize with PIFs (Hao, *et al.* 2012, Hornitschek, *et al.* 2009). It is therefore conceivable that these factors can function synergistically or additively in vivo.

Much work remains to elucidate the molecular mechanism underlying phytochrome inhibition of DNA binding of PIFs. We have shown that the inhibition requires a light-dependent interaction between phytochrome and PIFs and can occur in the absence of ATP. This indicates that the interaction itself is sufficient to disrupt DNA binding without phosphorylation. This implies that the interaction between PIFs and phyB-Pfr can directly compete with DNA binding. We speculate that this association induces an allosteric change in PIFs to destabilize DNA binding. Our data, however, do not exclude the possibility that the phosphorylation of PIFs and/or the interaction with other proteins such as DELLA can further destabilize the interaction between PIFs and their target promoters in vivo. A recent

report indicated that the DNA binding of PIF7, which is phosphorylated, but not destabilized by high R:FR, is also inhibited by high R:FR in vivo (Li *et al.* 2012). This suggests that the inhibition of PIF DNA binding by phytochrome might be a common mechanism for other PIFs. Further studies are needed to determine the detailed molecular mechanism of how phytochrome disrupts DNA binding of PIFs.

Experimental Procedures

Plant materials and growth conditions

Arabidopsis thaliana plants were maintained in a controlled growth facility with a 16-h light/8-h dark cycle at 22 to 24°C for general growth and seed harvesting. The *NG-GUS-NLS* (*NGB*) line (*NG-GUS-NLS 6-4*) expressing the N-terminal domain of phyB fused to GFP, GUS, and NLS in *phyB-5* null background in the Ler ecotype was reported previously (Matsushita, *et al.* 2003). *PIF3-OX* and *PIF1-OX* correspond to previously described *PIF3-OX3* and *PIL5-OX3* (Oh, *et al.* 2006, Park, *et al.* 2004). Two independent *NGB/PIF3-OX/phyB* lines were established by crossing *PIF3-OX/phyB-9* with *NGB/phyB-5*. Four independent *NGB/PIF3-OX/phyA/phyB* lines were isolated by crossing *PIF3-OX/phyA-211* with *NGB/PIF3-OX/phyB-9*. All showed similar partial phosphorylation and no degradation of PIF3. *PIF3ΔN* lines that express myc-tagged PIF3 lacking the N-terminal 300 amino acids under 35S promoter were generated by cloning N-terminal deleted *PIF3* to pHTM vector using a specific primer sets (Table S1), transforming, and selecting homozygous lines in Col-0 background.

Chromatin Immunoprecipitation (ChIP)

For the ChIP analysis, seedlings were grown under dark or red light ($10 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 4 days. For some experiments, four-day old seedlings were treated with $80 \mu\text{M}$ MG132 before sampling for 8 hours. Samples (1 g) of 4 day-old dark or red light grown seedlings were crosslinked with 10 ml of 1% formaldehyde under vacuum infiltration conditions. ChIP assays were performed as described previously (Shin *et al.* 2007) with a minor modification of the three wash buffers: Low wash buffer (150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8), High Salt Wash buffer (500 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8), LiCl Wash Buffer (0.25 M LiCl, 1% v/v NP-40, 1% w/v sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8). The amount of each precipitated DNA fragment was determined by real-time PCR using *RGA*, *PIL1*, and *rDNA* primers (Table S1).

Phosphorylation and degradation of PIF3

For the calf intestine alkaline phosphatase (CIP) treatments, 5-day-old dark grown seedlings expressing his- or myc-tagged PIF3 were ground in liquid nitrogen and homogenized in a denaturing buffer (100 mM NaH_2PO_4 , pH 8.0, 10 mM NaCl, 8 M urea, 2 mM PMSF, $80 \mu\text{M}$ MG132, 1x complete protease inhibitor cocktail (Roche, USA). After centrifugation at 14500 rpm for 10 min at 4 °C, PIF3 was purified from supernatants using Ni-NTA beads (Qiagen, Germany). Pellets were washed twice with PBS buffer ($8 \text{ g NaCl liter}^{-1}$, $0.2 \text{ g KCl liter}^{-1}$, $1.44 \text{ g Na}_2\text{HPO}_4 \text{ liter}^{-1}$, $0.24 \text{ g KH}_2\text{PO}_4 \text{ liter}^{-1}$, pH 7.4) and once with CIP buffer (NEBuffer 3: 100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl_2 , 1mM DTT, pH 7.9). The resuspended pellets were treated for 15min at 37°C with no enzyme, 100 U calf-intestinal alkaline phosphatase (CIP, NEB), or comparable amounts of boiled CIP. Following these incubations, reaction mixtures were boiled in 2xSDS sample buffer (45 mM Tris-HCl pH 6.8, 10% v/v glycerol, 1% w/v SDS, 0.05% w/v Bromophenol Blue, 50 mM DTT) and subjected to Western blot analysis with anti-myc antibody. For PIF protein stability, dark and light-grown 80 seedlings were frozen using liquid nitrogen and then homogenized in the

denaturing buffer (100 mM NaH₂-PO₄, 10 mM Tris-HCl, 8 M urea pH 8.0). The protein extraction and western blot were performed as described previously (Park, *et al.* 2004).

Co-immunoprecipitation assay

For the *PHYB-GFP* line, the pelleted nuclei fraction was resuspended in extraction buffer (without sucrose and with 0.5% Triton X-100) and immunoprecipitated with myc antibody. For the co-immunoprecipitation of phyB and PIF3, total proteins were extracted with an IP buffer (100 mM NaH₂PO₄, pH 7.8, 100 mM NaCl, 0.1% v/v NP-40, 2 mM PMSF, 100 μM MG132, 1x complete protease inhibitor cocktail). After removing debris by centrifugation at 14500rpm for 10 min at 4°C, the supernatant was pre-cleared with Protein A beads (Thermo Scientific, USA) at 4°C for 1 h to remove non-specific binding proteins and immunoprecipitated with anti-myc antibody (Santa Cruz Biotechnology, USA). To test the reversible binding of NGB with PIF3, the pre-cleared extracts from dark-grown seedlings were exposed to red light (10 μmol m⁻² s⁻¹) or far-red light (3 μmol m⁻² s⁻¹) for 20 min prior to co-immunoprecipitation.

DNA pull-down assay

Biotin-labeled double strand oligonucleotides corresponding to PIF-binding site of PIL1 promoter fragment (PIL1p, 300ng) were first bound to 20 μl of streptavidin agarose resin (Thermo Scientific). The resin was then equilibrated with 1 ml TKMG buffer (50mM Tris-HCl, pH 7.5/150mM KCl/1mM EDTA/5mM MgCl₂/0.5% (v/v) NP-40/10% (v/v) Glycerol) by washing three times. His-tagged PIF1 (200ng) or PIF3 (100ng) proteins were incubated with the PIL1p resin in the presence of 1μg poly dI-dC (Sigma) and 10μg BSA (NEB) at 4°C for 2h. Unbound PIF1 or PIF3 proteins were removed by washing three times with 1 ml of TKMG buffer. The PIF-bound PIL1p resin was incubated with 1μg of purified recombinant phyB at 4°C for 2h in the dark. PhyB was irradiated with far-red light (3 μmol m⁻² s⁻¹) for 10 min (Pr) or with red light (10 μmol m⁻² s⁻¹) for 10 min (Pfr) prior to the incubation. After washing three times with 1 ml of TKMG buffer under green light, bound PIFs were eluted by boiling in 2XSDS buffer. The eluted PIF1 or PIF3 proteins were resolved on SDS polyacrylamide gels, transblotted to nitrocellulose membrane, and detected by PIF1 or PIF3 antibody after running of SDS-PAGE. PIF3 antibody is a polyclonal antibody against PIF3-GST and PIF1 antibody is a polyclonal antibody against a PIF1 peptide (KTNVDDRKRKEREATT).

Recombinant full-length or N-terminal phytochrome B (aa1-650) preparations were obtained by cloning into pBAD vector, co-transformation with pPL-PCB into *E.coli* strain LMG 194, and purification as described (Gambetta and Lagarias 2001). For the purification, cells were collected by centrifugation, resuspended, and sonicated in a lysis buffer (50mM NaH₂PO₄, pH 8.0/300mM NaCl/10% Glycerol/20mM Imidazole/0.05% Tween-20/1mM 2-mercaptoethanol/1mM PMSF/1Xcomplete protease inhibitor cocktail (Roche)). Proteins were further purified with Ni²⁺-NTA agarose resin as described before (Gambetta and Lagarias 2001). Purified recombinant full length phyB bound PIF3 light-dependently (Figure S2).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

NGB does not degrade PIF3. **(A)** PIF3 was not degraded by NGB under continuous red light. Protein extracts were made from 4-day-old dark- and Rc-grown seedlings. PIF3 and tubulin were detected with anti-myc and anti-tubulin antibodies, respectively. NGB indicates the N-terminal domain of phyB fused to GFP, GUS, and a NLS. **(B)** Phosphorylation of PIF3 mediated by NGB under dark-to-red transfer conditions. His- and myc-tagged PIF was purified by Ni²⁺-NTA resin from five day old seedlings grown in the dark (Dc) or exposed to red light ($10 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 20 min (R20). Phosphorylation of PIF3 by red light was visualized as slow migrating bands that disappear following CIP treatment (+), but not following boiled CIP treatment (b). Multiple slow migrating phosphorylated bands were indicated sequentially by * and **. The lower panel is an independent dataset showing the partial phosphorylation.

Figure 2.

NGB inhibits the binding of PIF3 to its target promoter in vivo. **(A)** The Pfr form of NGB interacts with PIF3. Input indicates samples before immunoprecipitation and IP indicates co-IPd samples under white light. The interaction was also determined by irradiating with red light (R) or far-red light (FR) before co-IP. The * indicates non-specific band detected by anti-GFP antibody in total plant extracts. **(B)** Decreased binding of PIF3 to *RGA* and *PIL1* promoter fragments in red light grown seedlings determined by ChIP. The co-IPd DNA was expressed as relative values of *NGB/PIF3-OX/phyB*, Dc (SD, n=2, biological replicates). Dc indicates dark-grown *NGB/PIF3-OX/phyB* and Rc indicates red light-grown *NGB/PIF3-OX/phyB*. An rDNA fragment was used as a non-binding control. **(C)** Similar levels of PIF3 protein in Dc and Rc samples after the chromatin immunoprecipitation (After IP). Dc and Rc indicate protein extracts from 4-day-old dark-grown (Dc) or red light-grown (Rc) seedlings. **(D)** Decreased expression of *PIL1* mRNAs by red light in wild type (WT), the *NGB/phyB* line, *NGB/PIF3-OX/phyB* line, and *phyB* mutant. Relative expression levels of *PIL1* mRNAs were normalized by *PP2A* mRNA levels and by corresponding dark levels ($(PIL1_{Rc}/PP2A_{Rc})/(PIL1_{Dc}/PP2A_{Dc})$) (SD, n=2, biological replicates).

Figure 3.

Red light inhibits the binding of PIF3 and PIF1 to their target promoters in plants possessing full-length phyB. **(A)** The binding of PIF3 to *PIL1* and *RGA* promoters in Dc and Rc samples of the *PIF3-OX* determined by ChIP. The rDNA fragment was used as a non-binding control. The co-IPd DNA was expressed as relative values of *PIF3-OX*, Dc (SD, n=2, biological replicates). Dc and Rc indicate dark-grown and red light-grown *PIF3-OX* respectively. The upper panel indicates PIF3 protein levels after the ChIP (After IP). **(B)** MG132 treatment increases PIF3 protein levels both in Dc- and Rc-grown samples, but the binding of PIF3 to its target promoters increases only in Dc-grown *PIF3-OX*. DMSO is dimethyl sulfoxide used to dissolve MG132. The co-IPd DNA was expressed as relative values of *PIF3-OX*, Dc+DMSO (SD, n=2, biological replicates). **(C)** MG132 treatment increases PIF1 protein level and the subsequent binding of it to its target promoter in Dc-grown, but not in Rc-grown *PIF1-OX*. The co-IPd DNA was expressed as relative values of *PIF1-OX*, Dc+DMSO (SD, n=2, biological replicates). **(D)** A far-red pulse followed by a short dark incubation increases PIF3 and PIF1 protein levels mildly but the binding of them to their target promoter increases strongly in *PIF3-OX* and *PIF1-OX*. Red light-grown seedlings were irradiated with far-red light pulse (5'FR, $3 \mu\text{mol m}^{-2} \text{s}^{-1}$), transferred to dark for 10 (10'Dark) or 30 minutes (30'Dark), and sampled for ChIP analysis. The co-IPd DNA was expressed as relative values of either *PIF3-OX*, Rc, or *PIF1-OX*, Rc (SD, n=2, biological replicates).

Figure 4.

PhyB must be able to bind to PIF3 to inhibit interaction with its target promoter. **(A)** Red light does not decrease the binding of PIF3 to its target promoter in the absence of phyB as determined by ChIP. The rDNA fragment was used as a non-binding control. The co-IPd DNA was expressed as relative values of *PIF3-OX/phyB*, Dc (SD, n=2, biological replicates). Dc indicates dark-grown *PIF3-OX/phyB* and Rc indicates red light-grown *PIF3-OX/phyB*. The upper panel indicates PIF3 protein levels after the ChIP (After IP). **(B)** N-terminal deleted PIF3 that cannot interact with phyB (*PIF3ΔN*) binds to its target promoter equally well both in Dc and Rc conditions as determined by ChIP. The co-IPd DNA was expressed as relative values of *PIF3ΔN*, Dc (SD, n=2, biological replicates).

Figure 5.

Both full-length and N-terminal-domain phyB inhibit the binding of PIF1 and PIF3 to their target promoter in vitro. **(A)** Full-length phyB inhibits the binding of PIF1 and PIF3 to their target promoter in vitro. A biotinylated double-strand oligomer of the *PIL1* promoter (which possesses a PIF-binding site) and recombinant PIF proteins were mixed, and the DNA-protein complexes were separated and incubated with recombinant phyB in either the Pr form or the Pfr form. DNA was precipitated (IP) and bound PIF proteins were detected by antibodies against PIF1 or PIF3. **(B)** The N-terminal domain of phyB (N-phyB) inhibits the binding of PIF1 and PIF3 to their target promoter in vitro. Numbers indicate relative band intensities. **(C)** Quantification of PIF1 and PIF3 protein levels in (A) and (B) (SD, n=2, biological replicates).