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Photoactivated Phytochrome Induces Rapid PIF3 Phosphorylation Prior to Proteasome-Mediated Degradation

Short Article

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Summary

Following light-induced nuclear translocation, specific members of the phytochrome (phy) photoreceptor family (phyA to phyE) interact with bHLH transcription factors, such as PIF3, and induce changes in target-gene expression. The biochemical mechanism comprising signal transfer from phy to PIF3 has remained undefined but results in rapid degradation of PIF3. We provide evidence that photoactivation of phy induces rapid in vivo phosphorylation of PIF3 preceding degradation. Both phyA and phyB redundantly induce this PIF3 phosphorylation, as well as nuclear speckle formation and degradation, by direct interaction with PIF3 via separate binding sites. These data suggest that phy-induced phosphorylation of proteins such as PIF3 may represent the primary intermolecular signaling transaction of the activated photoreceptor, tagging the target protein for proteosomal degradation, possibly in nuclear speckles.

Introduction

The phytochrome (phy) family of sensory photoreceptors (phyA through phyE in *Arabidopsis*) monitor incident light signals and direct adaptational growth and developmental responses in plants appropriate to the prevailing environment (Schäfer and Nagy, 2006). The phy molecule is a soluble chromoprotein composed of a polypeptide of about 125 kilodaltons and a covalently linked tetrapyrrole chromophore (Tu and Lagarias, 2005). The photosensory function of the molecule resides in its capacity for reversible interconversion between the biologically inactive and active conformers, Pr and Pfr, respectively, upon sequential absorption of red (R) and far-red (FR) photons (Quail, 2002). The holoprotein is synthesized in the inactive Pr form in the cell and remains in the cytoplasm in dark-grown seedlings until exposure to light. R light perception (Pfr formation) initiates an intracellular signaling process that culminates in the induction or repression of target nuclear genes, detectable within 5 min of irradiation (Lissemore and Quail, 1988; Monte et al., 2004; Quail, 2002). This primary signaling process can be abrogated by subsequent FR light perception (reconversion to Pr) if occurring early enough to remove the active conformer before induction of the response. Evidence from comprehensive phy:GFP fusion-protein experiments has established that the phy molecule translocates rapidly into the nucleus upon light-induced Pfr formation, where it accumulates in numerous subnuclear bodies, called speckles (Kircher et al., 2002; Nagatani, 2004). This nuclear translocation has been shown to be necessary for the biological function of the photoreceptor (Huq et al., 2003; Matsushita et al., 2003).

The constitutively nuclear bHLH transcription factor PIF3 was identified in an early yeast two-hybrid screen as a phy interactor, and in vitro binding assays showed that phyA and phyB are induced by light to interact specifically in the Pfr form with this protein (Martínez-García et al., 2000; Ni et al., 1999). In the case of phyB, this interaction was shown further to require a specific N-terminal sequence motif in PIF3, designated APB (active phyB binding), which is also conserved in other PIF3-related bHLH proteins (Khanna et al., 2004). Coexpression of phyB:YFP and PIF3:CFP demonstrated the light-induced production of, and colocalization of phyB and PIF3 within, the abovementioned subnuclear speckles (Bauer et al., 2004). These data, together with *pif3* mutant studies (Monte et al., 2004), established PIF3 as a likely primary phy-signaling partner. However, the molecular basis of this signaling process was not apparent from these studies.

A pivotal observation by Bauer et al. (2004) provided important insight into the potential nature of the molecular transaction between phy and PIF3 in the cell. Continuous R light (Rc) was found to induce rapid degradation of the PIF3 protein in etiolated seedlings in a phy-dependent manner. A subsequent study showed that PIF3 degradation was inhibited by the proteasome inhibitor, MG132, and provided suggestive evidence of R-induced ubiquitination of PIF3 (Park et al., 2004). To address the question of the transmolecular signaling mechanism underlying the induction of phy-triggered PIF3 degradation, we focused here on examining the earliest molecular changes detectable in the properties of PIF3 after Pfr formation in the cell.

Results and Discussion

To approach this question, we developed antibodies against the low-abundance native PIF3 protein, as well as a series of transgenic *Arabidopsis* lines expressing various fluorescently labeled and epitope-tagged PIF3-fusion-protein constructs. Initial Western blot analysis with single, double, and triple *phy* null mutants confirmed and extended the findings of Bauer et al. (2004) that immunochemically measurable native PIF3 protein drops below the level of detection in wild-type (wt) seedlings within 60 min of initial exposure to Rc, and that phyA, phyB, and phyD act qualitatively redundantly in

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this process (see Figures S1A–S1C in the Supplemental Data available with this article online), but that phyD functions apparently less efficiently than phyA or phyB (Figure S1B). We found also that a brief (30 s) saturating (3000 μmol.m^{-2}) pulse of R light (Rp) is as effective as Rc in inducing PIF3 degradation and that an immediately subsequent long-wavelength, FR light pulse (FRp) abrogated this Rp-induced degradation, either partially in wt, or fully in the phyA mutant (Figure S1D). These data establish that the Pfr form of the photoreceptor functions in the dark to induce PIF3 degradation, that Pfr must be present for a finite period in the cell to execute this function, and that induction of PIF3 degradation is highly sensitive to extremely low levels of Pfr, being half-maximally induced by the Pfr level (0.1% of total phyA) established by long-wavelength FR light alone (Figure S1D).

Most strikingly, however, we noted a rapid shift in the electrophoretic mobility of PIF3 with increasing time of irradiation of seedlings with Rc before extraction (Figure 1A). This shift is detectable within 2 min of the onset of Rc irradiation (Figure 1A, inset) and is effectively induced by a brief, saturating Rp followed by darkness (Figure 1B). This Rp induction is fully blocked by an immediately subsequent long-wavelength FRp, which alone has no inductive effect. However, interposing a dark period of increasing duration between the Rp and the FRp results in induction of the PIF3 mobility shift within 60 s (Figure 1B). *phy* mutant analysis shows that phyA and phyB act essentially fully redundantly in this mobility shift induction but that phyD alone is ineffective (as are phyC and phyE) (Figure 1C). Time course analysis of the mobility shift and degradation of PIF3 in the *phyAphyB* double mutant shows that, although no mobility shift occurs in the mutant, degradation is observed over the 60 min period, albeit at a reduced rate compared to wt (Figure 1D). Treatment of seedlings with the proteasome inhibitor, MG132, inhibits the Rc-induced degradation of PIF3, but not the mobility shift, resulting in the retention of the lower-mobility species over a 1 hr Rc irradiation period (Figure 1E). In addition to confirming evidence that degradation appears to occur via the proteasome system (Park et al., 2004), these data establish that the mobility shift is not a product of the proteasomal degradation process per se but is rather the product of an upstream event. We interpret these data to indicate: (1) that the Pfr form of phyA and phyB induces a molecular alteration in PIF3 as rapidly as 1 min following photoactivation in the cell, (2) that the mobility shift precedes degradation and may be a prerequisite for it, and (3) that the reason that neither phyD nor the low levels of phyA formed by long-wavelength FR light alone can induce a mobility shift in PIF3 is that the absolute rate of degradation exceeds the absolute rate of mobility shift generation in these cases, thereby precluding accumulation of altered, but undegraded, PIF3.

In principle, the mobility shift in PIF3 could be the result of any of a number of light-induced intracellular molecular modifications, including ubiquitination (Park et al., 2004) or phosphorylation (Duek et al., 2004; Lipford and Deshaies, 2003; Sasaki et al., 2003). The data in Figure 2 show that the Rp-induced mobility shift observed at 10 and 15 min following pulse irradiation is abolished by in vitro treatment of the PIF3 preparations with calf-intestinal alkaline phosphatase (CIAP), but not if the CIAP enzyme is first boiled to inactivate it. This reversal of the shift was observed with PIF3 extracted under denaturing (Figure 2B) or nondenaturing (Figure 2C) conditions. The data strongly suggest, therefore, that the mobility shift observed on gels in vitro is the result of rapid, light-induced, intracellular phosphorylation of PIF3 prior to extraction.

To determine whether the direct interaction of photoactivated phy with PIF3, observed by in vitro interaction assay, is necessary for the phosphorylation-based mobility shift induced in the cell, we sought to construct and transgenically express PIF3 protein variants specifically lacking phyA and phyB binding activity. Previously, we showed that the mutation of two conserved residues, E31A and G37A, within the APB domain of PIF3 completely eliminated the Pfr-specific binding of phyB to the molecule (Khanna et al., 2004). Surprisingly, however, neither deletion nor mutation of the APB motif affected phyA binding to PIF3 (Figure 3A, left panel). This led us to identify a second site (designated APA for Active phyA) within the PIF3 protein that is necessary for Pfr-specific phyA binding (Figure 3A, right panel). Site-directed substitution of two residues, F203A and F209A, within this motif completely eliminated detectable phyA binding to PIF3. We transgenically expressed, in a *pif3* null mutant background, full-length YFP:PIF3-protein variants carrying these respective amino acid substitutions, either singly or combined, in mutant APB (mAPB) and/or mutant APA (mAPA) motifs, under control of the native *PIF3* gene promoter. Evidence that the fusion protein is biologically active in these transgenic lines is presented in Figure S2B. Figure 3B shows that the rapid, Rp-induced mobility shift observed in the wt configuration of this fusion protein is fully retained in the mAPB (non-phyB binding) variant and to a somewhat reduced extent in the mAPA (non-phyA binding) variant. Compellingly, however, this shift is eliminated in the mAPBmAPA double mutant PIF3 variant (two independent transgenic lines shown, Figure 3B). These data strongly suggest that direct, Pfr-specific binding of phyA or phyB to PIF3 is necessary in the cell to induce the observed phosphorylation of PIF3. Time course analysis demonstrates further that light-induced degradation of PIF3 is eliminated in the mAPBmAPA double mutant protein (Figure 3C). These data are consistent with the conclusion that phy-induced phosphorylation of PIF3 may be a necessary prerequisite for subsequent degradation, although the possibility that both phosphorylated and unphosphorylated forms of PIF3 are degraded cannot be excluded by the present data.

Previous data have shown that both phyA and phyB colocalize rapidly with PIF3 in nuclear speckles and that the presence of PIF3 is necessary for phyB speckle formation (Bauer et al., 2004). To determine whether direct intranuclear phy-PIF3 interaction is necessary for PIF3 speckling and degradation, we examined the cytological behavior of the YFP:PIF3 fusion variants in the same transgenic lines analyzed by Western blot above. The data show that, consistent with previous reports (Bauer et al., 2004; Nagatani, 2004), the wt PIF3 protein forms nuclear speckles within 1 min of Rp irradiation, followed by a decline in levels of visually detectable fluorescence over the next 5–10 min (Figure 4A, top panels).

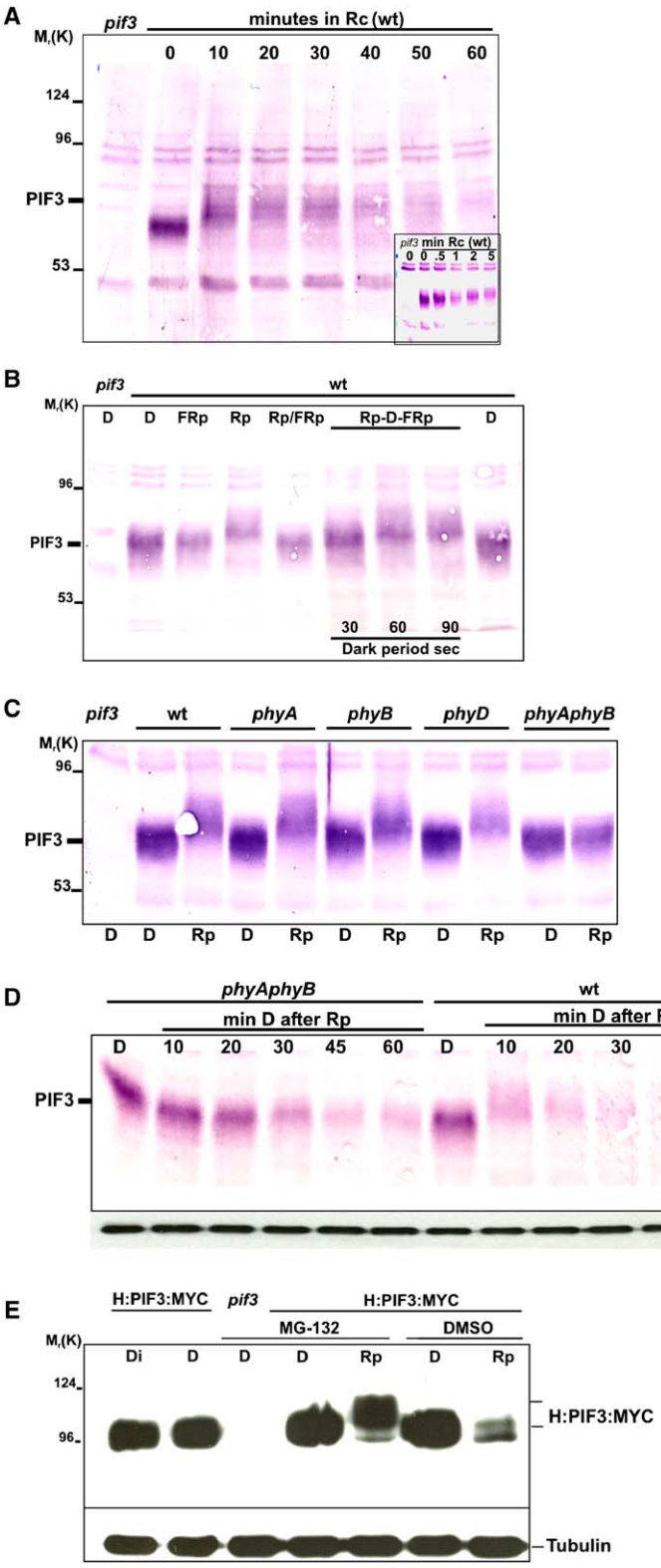


Figure 1. Photoactivated phys Induce a Rapid PIF3 Mobility Shift Prior to Proteasome-Mediated Degradation

(A–D) Western blot analysis of endogenous PIF3 extracted directly into denaturing buffer from seedlings grown for 4 days in the dark and then either maintained in darkness (D) or subjected to various light treatments, as indicated, before extraction. Blotted samples were probed with affinity-purified antisera against PIF3. (A) Time course of PIF3 mobility shift and degradation in wild-type (wt) seedlings irradiated with Rc at 10 μmol $m^{-2}s^{-1}$ for the periods indicated. *pif3* null mutant shown as negative control. (Inset) Early time course (over 5 min) showing rapidity of shift. (B) Induced mobility shift escapes rapidly from reversibility by far-red light. Dark-grown (D) wt seedlings were exposed to a saturating pulse (3000 μmol $m^{-2}s^{-1}$) of 30 s of R (Rp), a long-wavelength far-red (RG9) pulse (FRp) alone, or a Rp followed by FRp, either immediately (Rp/FRp) or after a brief interposed dark period of 30, 60, or 90 s (Rp-D-FRp), followed by return to darkness before extraction at 10 min from the initial Rp. (C) phyA and phyB, but not phyD, act redundantly in inducing the rapid PIF3 mobility shift. wt, *pif3*-null, *phy* monogenic, and *phyAphyB* double mutant were kept in darkness (D) or exposed to a 30 s saturating Rp and returned to darkness for 10 min before extraction. (D) R-induced PIF3 degradation rate is slower in *phyAphyB* double mutant than wt. Dark-grown seedlings (D) were exposed to a 30 s saturating Rp and returned to the dark for the periods indicated before extraction.

(E) The proteasome inhibitor, MG132, inhibits R-induced PIF3 degradation leading to accumulation of the modified PIF3 species. Three day dark-grown transgenic seedlings expressing 35S::6×HisPIF3:5×MYC (H:PIF3:MYC) were pretreated for 4 hr with either MG132 or DMSO and then kept in darkness (D) or exposed to a 30 s saturating Rp and transferred back to the dark for 1 hr before extraction and Western blot analysis using antibodies against the MYC epitope or tubulin as a control. MG132-treated *pif3* mutant seedlings served as negative control. Di, protein extraction immediately after 3 days dark growth without further treatment.

Similar behavior is observed for the mAPB (non-phyB binding) variant. Significantly reduced speckling and slower disappearance of fluorescence is observed for the mAPA (non-phyA binding) variant. Most strikingly, however, no detectable light-induced PIF3 speckling was observed for the mAPBmAPA double-mutant derivative and no detectable degradation over the period investigated (Figure 4A, bottom panels). These data indicate (1) that direct interaction of PIF3 with photoactivated phyA or phyB is indeed necessary for both early speckle formation and degradation, (2) that phyA might be somewhat more efficient in this process than phyB,

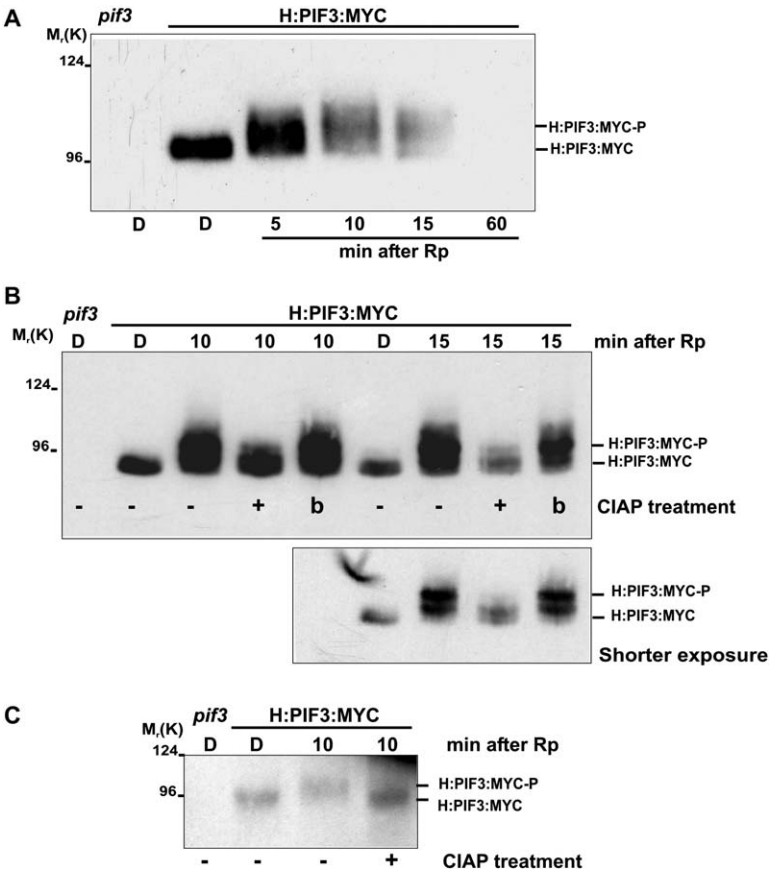


Figure 2. Red Light Induces Rapid PIF3 Phosphorylation In Vivo

Western blot analysis of epitope-tagged PIF3 extracted from transgenic seedlings expressing 6×His:PIF3:5×MYC (H:PIF3:MYC) grown for 4 days in the dark and then either maintained in darkness (D) or exposed to a 30 s saturating red light pulse (Rp) and returned to the dark for the periods indicated before extraction. Blotted samples were probed with antibodies against the MYC epitope. (A) Epitope-tagged PIF3 undergoes rapid Rp-induced mobility shift and degradation. Seedlings were extracted directly in denaturing buffer for Western blot analysis. (B and C) PIF3 mobility shift is reversed by alkaline phosphatase treatment. (B) H:PIF3:MYC was isolated from seedlings maintained in the dark (D) or exposed to a Rp followed by return to darkness for 10 or 15 min before extraction into denaturing buffer. The tagged PIF3 protein was affinity purified with Ni^{+}-NTA beads and either treated (+) or not (−) with alkaline phosphatase or with heat-inactivated alkaline phosphatase (b) before Western blot analysis. CIAP, calf intestine alkaline phosphatase, H:PIF3:MYC-P, phosphorylated H:PIF3:MYC species. (C) Seedlings treated as in (B), but extracted into nondenaturing buffer. The tagged PIF3 protein was then affinity purified with anti-MYC beads and subjected to alkaline phosphatase treatment as in (B). Evidence is presented in Figure S2A that the H:PIF3:MYC fusion protein is biologically active.

and (3) that speckle formation may be a prelude to and/or be directly involved in the degradation process. Moreover, the cytological behavior of the mutant YFP:PIF3 variants (Figure 4A) is qualitatively parallel to that observed by Western blot analysis (Figure 3). Collectively, then, these data suggest that the phy-induced phosphorylation of PIF3 may initiate rapid migration to nuclear speckles that may function as sites of ubiquitination and/or proteasomal degradation (Figure 4B). Alternatively, simple, physical phy-PIF3 interaction might induce comigration to these speckles where phosphorylation, ubiquitination, and degradation all occur.

The data presented here suggest that the induced intracellular phosphorylation of proteins such as PIF3 may represent the primary biochemical mechanism of phy signaling, in this case providing a molecular signal that flags the transcription factor for ubiquitination and degradation. Although the constitutively cytoplasmically localized protein PKS1 (Fankhauser et al., 1999) has been reported to be phosphorylated in seedlings exposed to prolonged (4 days) Rc, there is no direct photobiological or genetic evidence that the phy system mediates this long-term effect, there is no evidence that this modification alters the in vivo activity or molecular behavior of the PKS1 protein, and, importantly, there is no evidence that this modification is functionally relevant to light signaling in the cell (Fankhauser et al., 1999). The identification of rapid PIF3 phosphorylation provides the first direct evidence that phy-mediated transphosphorylation does indeed occur in the cell in a subcellular location previously established as a site of action necessary for phy signaling (Huq et al., 2003; Matsushita et al., 2003) and in a manner that requires direct, conformer-specific interaction between the activated photoreceptor molecule and its target.

Nevertheless, this proposed mechanism of phy signaling leaves several centrally important questions to be answered. First, is the phy molecule itself the presumptive protein kinase responsible for PIF3 phosphorylation? Such intrinsic kinase activity has long been proposed based on the evolutionary origins of the plant phys from the bacterial phys, which are members of the two-component histidine kinase superfamily (Montgomery and Lagarias, 2002), and biochemical evidence that purified preparations of plant phys contain serine-kinase activity capable of phosphorylating the phy molecule itself and certain other proteins, including PKS1 (Fankhauser et al., 1999). However, critical mutational analysis eliminating contaminating kinases as the source of the in vitro-measured enzymatic activity, and demonstrating the necessity of this activity to phy signaling in the living plant, is lacking (Quail, 2002). Indeed, Nagatani and colleagues have provided evidence that the entire C-terminal kinase-related domain of phy is dispensable for phy signaling in vivo (Matsushita et al., 2003). We have shown that phy preparations can phosphorylate PIF3 in vitro but have observed no compelling dependence on the Pfr form of the photoreceptor (data not shown). A second question is the identity of the presumptive E3-ligase responsible for the apparent ubiquitination of PIF3. An increasing number of major plant signaling systems have been shown to utilize induced

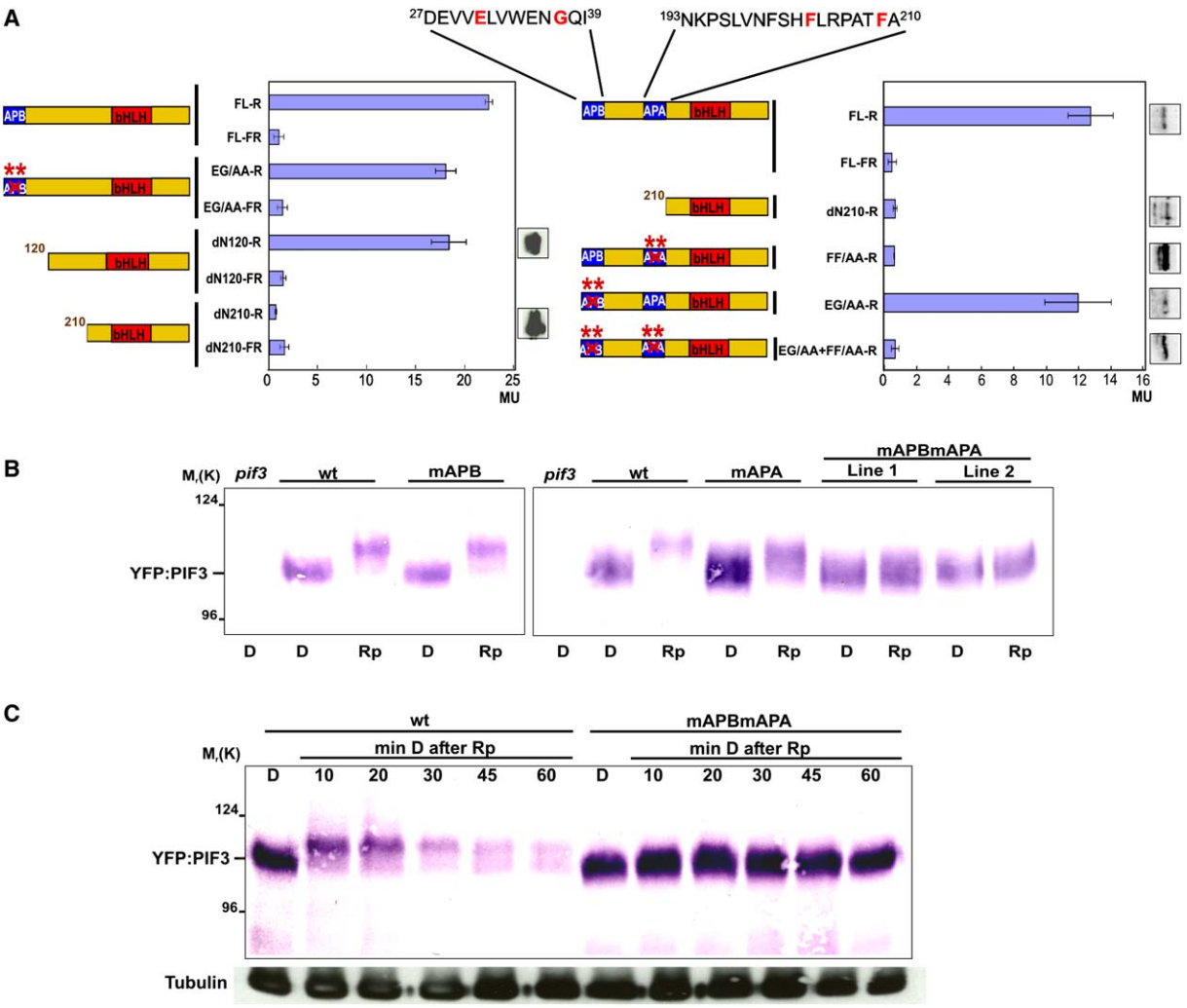


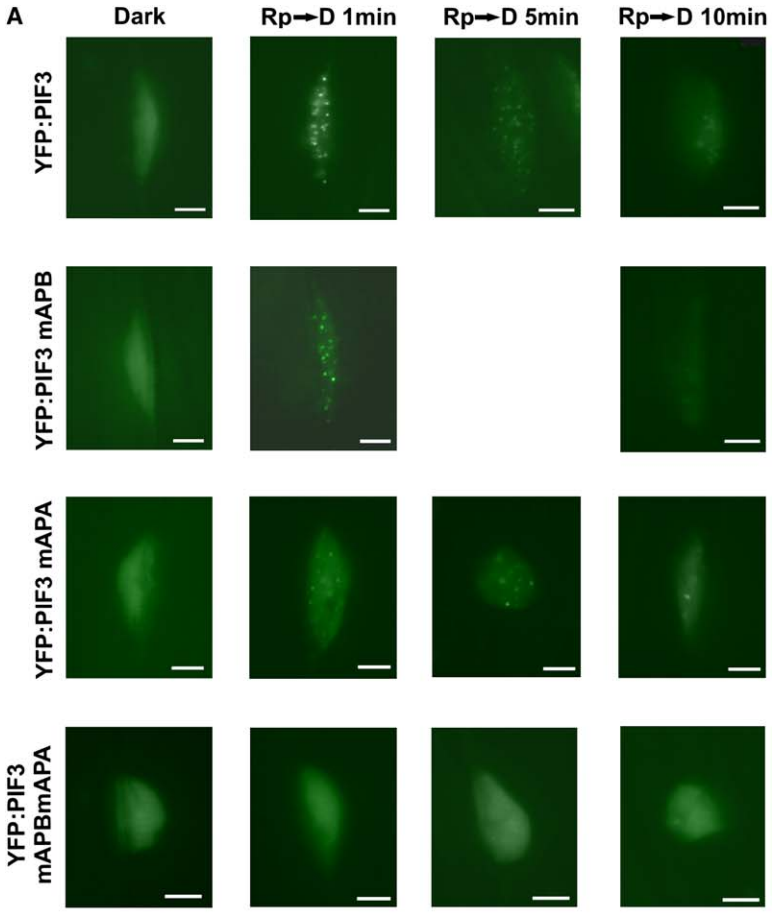
Figure 3. Direct Binding of phyA or phyB to PIF3 In Vivo Is Required for Phosphorylation and Degradation

(A) Photoactivated phyA binds to PIF3 at a separate site to phyB. R/FR light-reversible yeast two-hybrid assays were performed with phyA-DBD and full-length PIF3-AD or with various PIF3 deletion derivatives and point mutants. Transformed yeast were incubated in the presence of 25 μM phy chromophore overnight in darkness, exposed to a 1 min saturating Rp alone (R) or Rp followed by saturating far-red light (730 nm LED) pulse (FR), and diluted into YPD media. Anti-GAD antibody Western blots on yeast extracts are shown to the right for selected samples, verifying expression of the PIF3 fusion protein. EG/AA, site-directed mutations of PIF3 residues E31 and G37 to A; and FF/AA, mutations of F203 and F209 to A. APB and APA sequences are indicated with mutated residues in red. APB, active phyB binding region; APA, active phyA binding region; bHLH, basic helix-loop-helix domain; MU, miller units. Error bars represent standard error.

(B) Direct binding of photoactivated phyA or phyB to PIF3 is required for PIF3 phosphorylation in vivo. Seedlings of the *pif3* mutant or transgenic lines expressing various *pif3::YFP:PIF3* fusion constructs were grown for 4 days in darkness and then either maintained in the dark (D) or given a 30 s saturating Rp and returned to darkness for 8 min before extraction into denaturing buffer and Western blot analysis using affinity-purified antisera against PIF3. Wt, wild-type PIF3 protein sequence fused to the YFP protein. mAPB, YFP:PIF3 fusion protein carrying E31A and G37A mutations in the APB motif. mAPA, YFP:PIF3 fusion protein carrying F203A and F209A mutations in the APA motif. mAPBmAPA, YFP:PIF3 fusion protein carrying all four point mutations in both APB and APA motifs (two independent lines, line1 and line 2).

(C) Direct binding of photoactivated phyA or phyB to PIF3 is required for PIF3 degradation in vivo. Transgenic lines expressing the wild-type YFP:PIF3 (wt) or doubly mutated YFP:PIF3 (mAPBmAPA) fusion proteins described in (B) were maintained in darkness (D) or exposed to a 30 s saturating Rp and returned to the dark for increasing periods as indicated. Extraction and Western blotting as in (B).

protein degradation via the UPS system as a primary step in the signaling cascade (Dharmasiri et al., 2005; Sasaki et al., 2003; Smalle and Vierstra, 2004). Finally, the implications of the rapid degradation of PIF3 for its function as a transcriptional regulator remain to be assessed. One possibility is that PIF3 functions in dark-grown seedlings, either as a positive regulator of genes necessary for skotomorphogenesis or as a repressor of genes necessary for photomorphogenesis, and that light-induced degradation reverses this activity. An attractive alternative possibility is offered by the emerging evidence in other systems that the molecular modifications (phosphorylation, ubiquitination) integral to the induced degradation of certain transcription factors via the UPS system function to modulate the intrinsic transcriptional regulatory activity of the factor, transiently, before degradation (Lipford and Deshaies, 2003). This alternative raises the possibility that the phosphorylated or ubiquitinated form of PIF3 is transcriptionally activated prior to degradation.



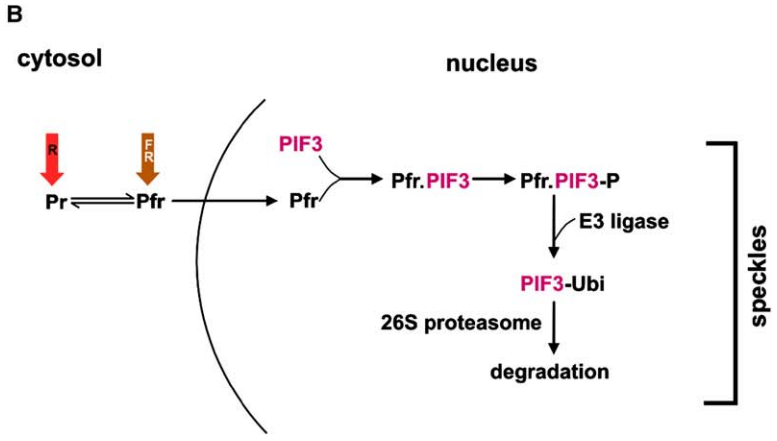


Figure 4. Direct phyA or phyB Binding to PIF3 Is Required for Rapid Light-Induced PIF3 Speckle Formation and Subsequent Degradation

(A) Epifluorescent imaging of YFP fluorescence in hypocotyl-cell nuclei of transgenic seedlings expressing the same various *pif3::YFP:PIF3* fusion constructs as in Figure 3. Seedlings were grown for 4 days in darkness and then either maintained in the dark (D) or given a 30 s saturating Rp and returned to darkness (D) for 1, 5, or 10 min before imaging. YFP:PIF3, wt-PIF3 protein sequence fused to the YFP protein;YFP:PIF3mAPB, YFP:PIF3 fusion protein carrying E31A and G37A mutations in the APB motif; YFP:PIF3 mAPA, YFP:PIF3 fusion protein carrying F203A and F209A mutations in the APA motif; YFP:PIF3mAPBmAPA, YFP:PIF3 fusion protein carrying all four point mutations in both APB and APA motifs. Scale bars indicate 5 μm.

(B) Model for signal transfer from photoactivated phy to PIF3. It is proposed that, following photoactivation and nuclear import, Pfr associates with PIF3, inducing rapid PIF3 phosphorylation (PIF3-P). PIF3-P is then recognized and ubiquitinated (PIF3-Ubi) by a yet-unidentified E3 ligase and is finally degraded by the 26S proteasome. Visually observed light-induced nuclear speckles could represent any of the proposed PIF3 processing events or foci where these events are all physically coupled.

Experimental Procedures

Plant Material

Col-O wild-type; *pif3-3* mutant in Col-O; or *phyA*, *phyB*, and *phyD* mutants or higher order combinations thereof, in L*er*, with *L*er wild-type control seeds were grown in darkness for 4 days at 21°C as described (Monte et al., 2004) before R or long-wavelength FR (RG9) light treatments as indicated (Ni et al., 1999; Wehmeyer et al., 1990). Pif3::YFP:PIF3 transgenic lines were generated as follows: the *PIF3* promoter was cloned from Col-O genomic DNA by PCR into pEZS-CL with the eGFP replaced with eYFP (Clontech). The PIF3 cDNA was cloned in frame to the eYFP. Point mutations in the PIF3 cDNA were introduced using a QuikChange Site-Directed Mutagenesis Kit (Stratagene). The *pif3::eYFP:PIF3* constructs were then moved into the binary vector pART27. *Pif3-3* plants were transformed with these constructs as described (Bechtold et al., 1993). 35S::6xHis:PIF3:5xMYC transgenic lines were generated as follows: *PIF3* cDNA was amplified with a forward primer containing 6×His and a reverse primer containing 1×MYC sequences. The product was cloned into pENTR/D-TOPO vector (Invitrogen) and transferred into a binary vector (pGWB17) containing a 35S promoter and 4XMYC fusion at the C terminus. *Pif3-3* plants were transformed with this construct as described (Bechtold et al., 1993). For MG132 treatments, 3-day-old dark grown seedlings were treated with 50 μM MG132 or 1% DMSO in liquid GM medium for 4 hr and washed three times in liquid GM medium before light treatments.

PIF3 Antibody Production and Immunoblots

A fragment of *PIF3* encoding amino acids 67–340 was amplified by PCR and cloned into pinpoint Xa-1 vector (Promega) in frame with the Xa-1 biotin binding domain tag. The fusion protein was expressed in BL21 *E. coli* cells and purified using Soft Link Avidin resin (Promega) according to manufacturer's instructions. Antisera were produced (Covance) and the crude serum was affinity purified using a GST-PIF3 (Martínez-García et al., 2000) affinity column. For immunoblots, seedlings were extracted into boiling extraction buffer (100 mM MOPS [pH 7.6], 40 mM 2-mercaptoethanol, 5% SDS, 10% glycerol 4 mM EDTA, 2 μgl-1 aprotinin, 3 μgl-1 leupeptin, 1 μgl-1 pepstatin, and 2 mM PMSF), 60–80 μg protein was separated on 6%–8% SDS-PAGE gels, and blots were probed with the purified PIF3 serum as primary antibody and alkaline phosphatase-coupled anti-rabbit antiserum (Promega) as a secondary antibody. For immunodetection of 6×His:PIF3:5×MYC, anti-MYC antisera (Covance) and a HRP-conjugated secondary antiserum (Promega) were used.

PIF3 Immunoprecipitation and Alkaline Phosphatase Treatments

Four-day-old transgenic seedlings expressing 35S::6×His:PIF3:5×MYC were frozen in liquid nitrogen and extracted at 3:1 v/w into either nondenaturing buffer (100 mM Na phosphate [pH 7.8], 100 mM NaCl, 0.5% Triton X-100, 25 mM NaF, 10 mM Na orthovanadate, 4 mM EDTA, 2 μgl-1 aprotinin, 3 μgl-1 leupeptin, 1 μgl-1 pepstatin, 2 mM PMSF, and 1× complete protease inhibitor cocktail [Boehringer Mannheim]) or denaturing buffer (100 mM Na phosphate [pH 7.8], 100 mM NaCl, 8 M urea, 2 μgl-1 aprotinin, 3 μgl-1 leupeptin, 1 μgl-1 pepstatin, and 2 mM PMSF and 1× complete protease inhibitor cocktail [Boehringer Mannheim]) and cleared by centrifugation at 16000 × g for 10 min. PIF3 was immunoprecipitated from supernatants with anti-MYC affinity matrix (Covance) or Ni^{2+}-NTA beads (Qiagen) following incubation for 4 hr at 4°C. The pellets were washed twice with PBS wash buffer as described (Ni et al., 1999) and once with CIAP buffer (100 mM Tris [pH 9.0], 50 mM MgCl2, 100 mM NaCl, and 1× complete protease inhibitor cocktail [Boehringer Mannheim]). The pellets were resuspended in 50 μl CIAP buffer and then treated for 15 min at 37°C with either no enzyme, 100 U calf-intestinal alkaline phosphatase (CIAP, Roche), or the same amounts of heat-inactivated CIAP. Pellets were boiled in 1× SDS-Laemmli buffer and subjected to Western blot analysis with anti-MYC antisera as above.

Yeast Two-Hybrid Mapping of PIF3-phyA Interaction Sites

Quantitative yeast two-hybrid interaction assays with phyBNT:DBD, phyAFL:DBD, and PIF3:GAD were performed as described (Shimizu-Sato et al., 2002). PIF3 deletions were constructed using forward primers containing EcoRI sites and reverse primers containing BamHI sites and cloned into pGAD424 (Clontech). Point mutations in the PIF3 cDNA were introduced as above. Yeast extracts for Western blots were obtained as described (Clontech, Yeast Protocols Handbook). Western blots were performed using anti-GAD monoclonal antisera (Santa Cruz Biotechnology).

Epifluorescent Microscopy

Epifluorescence microscopy analysis was according to Bauer et al. (2004) by using an Axiovert 200 microscope (Zeiss, Oberkochen, Germany) with excitation and detection of YFP fluorophore using Zeiss fluorescence filter set 46 (Zeiss). Images were recorded with a digital Axiocam camera (Zeiss) and processed for optimal presentation using the Corel Photo Paint 10 (Corel Corp., Ottawa) software package.

Supplemental Data

Supplemental Data include two figures and can be found with this article online at http://www.molecule.org/cgi/content/full/23/3/439/DC1/.

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