Phytochrome A Antagonizes PHYTOCHROME INTERACTING FACTOR 1 to Prevent Over-Activation of Photomorphogenesis

Martín Krzymuski[a], Pablo D. Cerdán[b], Ling Zhu[c], Amanda Vinh[c], Joanne Chory[d], Enamul Huq[c], and Jorge J. Casal[a,b,1]

a IFEVA, Faculty of Agronomy, University of Buenos Aires, and Consejo Nacional de Investigaciones Científicas y Técnicas, Av. San Martín 4453, 1417-Buenos Aires, Argentina

b Fundación Instituto Leloir, IIBBA-CONICET, C1405BWE-Buenos Aires, Argentina

c Department of Molecular Biosciences and the Institute for Cellular and Molecular Biology, University of Texas, Austin, TX 78712, USA

d Plant Biology Laboratory, Howard Hughes Medical Institute, the Salk Institute for Biological Studies, La Jolla, CA 92037, USA

ABSTRACT **Phytochrome A (phyA) is crucial to initiate the early steps of the transition between skoto- and photomorphogenesis upon light exposure and to complete this process under far-red light (typical of dense vegetation canopies). However, under prolonged red or white light, *phyA* mutants are hyper-photomorphogenic in many respects. To investigate this issue, we analyzed the late response of the transcriptome of the *phyA* mutant to red light. Compared to the wild-type (WT), hyper-responsive genes outnumbered the genes showing reduced response to red light in *phyA*. A network analysis revealed the co-expression of *PHYTOCHROME INTERACTING FACTOR 1* (*PIF1*) with those genes showing hyper-promotion by red light in *phyA*. The enhanced responses of gene expression, cotyledon unfolding, hypocotyl growth, and greening observed in the *phyA* mutant compared to the WT were absent in the *phyA pif1* double mutant compared to *pif1*, indicating that the hyper-photomorphogenic phenotype of *phyA* requires PIF1. PIF1 directly binds to gene promoters that displayed PIF1-mediated enhanced response to red light. Expression of mutant PIF1 deficient in interactions with phyA and phyB enhanced the long-term growth response to red light but reduced the expression of selected genes in response to red light. We propose that phytochrome-mediated degradation of PIF1 prevents over-activation of photomorphogenesis during early seedling development.**

Key words: phyA; PIF1; de-etiolation; light (gene expression); light (growth).

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INTRODUCTION

Light initiates de-etiolation when aerial plant tissues emerge from the soil. In *Arabidopsis*, de-etiolation involves the reduction of the rate of extension growth of the embryonic stem (hypocotyl) that drives shoot emergence, the unfolding and expansion of the cotyledons, the synthesis of the photosynthetic apparatus (i.e. the transition to autotrophy), and the synthesis of photoprotective pigments (Chen et al., 2004; Kami et al., 2011).

In darkness, the photoreceptors phytochromes are synthesized in the inactive Pr form which is photo-converted to the active Pfr form to initiate de-etiolation (Li et al., 2011). A portion of the Pfr pool migrates to the nucleus (Toledo-Ortiz et al., 2010b; Pfeiffer et al., 2012) where one of the actions is to bind the transcription factors named PHYTOCHROME INTERACTING FACTORS (PIFs) (Castillon et al., 2007; Bae and Choi, 2008; Leivar and Quail, 2011; Leivar and Monte, 2014). PIF proteins are bHLH transcription factors that repress photomorphogenesis in darkness (Leivar et al., 2008; Shin et al., 2009). Binding of Pfr causes the phosphorylation, reduced DNA

[1] To whom correspondence should be addressed. E-mail casal@ifeva.edu.ar, fax 5411-4524-4040, tel. ++5411-4524-8070 ext. 8123.

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binding capacity by sequestration, and/or proteasome-mediated degradation of PIF proteins (Castillon et al., 2007; Leivar and Quail, 2011; Li et al., 2012; Park et al., 2012), resulting in promotion of photomorphogenesis. These events are only part of the complex signaling network involved in de-etiolation. This network includes cryptochromes in addition to phytochromes, cytoplasmic in addition to nuclear events, and the down-regulation of the E3-ligase CONSTITUTIVE PHOTOMORPHOGENIC 1 (which in darkness causes destruction of positively acting transcription factors necessary for photomorphogenesis) in addition to that involving PIF proteins (Chen et al., 2004; Kami et al., 2011).

Phytochromes are encoded by five genes (*PHYA* through *PHYE*) in *Arabidopsis thaliana* (Clack et al., 1994). The rate of Pr to Pfr phototransformation is maximal under red light and far-red light photo-converts Pfr back to Pr. There is some overlap in the absorption spectrum of Pr and Pfr and, therefore, far-red light establishes a small amount of Pfr, which in the case of phyA is biologically very significant. The most abundant phytochrome in darkness is phyA (Sharrock and Clack, 2002), which plays a predominant role in the early events of photomorphogenesis under red light including the inhibition of hypocotyl growth (Parks and Spalding, 1999) and the large reshaping of the transcriptome (Tepperman et al., 2006). Under prolonged red light, the contribution of phyA to photomorphogenesis decreases (Parks and Spalding, 1999) and phyB becomes more important (Quail et al., 1995). Prolonged red light rapidly reduces transcription of *PHYA* (Cantón and Quail, 1999) as well as induces the degradation of phyA Pfr in the proteasome (Clough and Vierstra, 1997) resulting in reduced activity of phyA. Under far-red light, phyA remains highly active during de-etiolation (Rausenberger et al., 2011; Possart et al., 2014) and this helps to complete the process under dense canopies (Casal et al., 2014).

Under prolonged red light, a residual contribution of phyA can be observed. This action is partially masked by phyA-mediated negative regulation of phyB signaling when both photoreceptors are activated simultaneously. In other words, under red light, phyA promotes de-etiolation and inhibits phyB-mediated de-etiolation (Mazzella et al., 1997; Cerdán et al., 1999; Franklin et al., 2007; Torres-Galea et al., 2013). Actually, the negative effect of phyA on de-etiolation can be stronger than its positive effect and the *phyA* mutant becomes more de-etiolated (e.g. shorter) than the wild-type (WT) (Mazzella et al., 1997). In tomato, phyA negative interference on phyB1 activity in the WT leads to approximately half the level of anthocyanin observed in the *phyA* mutant under red light (Weller et al., 2000). Experiments under natural radiation (that simultaneously activates phyA and phyB) have demonstrated that the negative effect of phyA on phyB-mediated inhibition of hypocotyl growth is quantitatively important (Mazzella and Casal, 2001; Casal et al., 2014).

The effect of phyA on phyB-mediated responses does not involve changes in PHYB protein levels and requires FHY1 (Cerdán et al., 1999), which facilitates phyA migration to the nucleus (Genoud et al., 2008). This suggests that this phyA-mediated regulation acts downstream of phyB in the nucleus. To investigate the mechanisms by which phyA is able to reduce the responses to red light, we studied the changes in gene expression under conditions where phyA-mediated reduction of the response to light dominates over phyA-mediated promotion of the responses to light. The results of these experiments provide insight into the functional significance of this dual effect of phyA and point to PIF1 as a key player in the interaction between phyA and phyB.

RESULTS

Enhanced Gene Expression Responses to Red Light in the *phyA* Mutant

One-day-old etiolated *Arabidopsis* seedlings were exposed for 3 d to a daily protocol consisting in a pulse of 5 min of red light and complete darkness the rest of the time (Figure 1A). Six hours after the last pulse, the seedlings were harvested for RNA extraction and hybridization to ATH1 Affymetrix microarrays. This protocol was used to focus on the negative regulation of photomorphogenesis by phyA. In effect, under one pulse of red light per day, the WT fails to unfold the cotyledons but the *phyA* mutant shows phyB-mediated cotyledon unfolding (absent in *phyA phyB*), indicating a strong negative effect of phyA on photomorphogenesis (Casal and Boccalandro, 1995; Yanovsky et al., 1997; Cerdán et al., 1999) (Figure 1B). We harvested 6 h after the third daily pulse of red light to elude the early positive contribution of phyA to photomorphogenesis (Parks and Spalding, 1999; Tepperman et al., 2006) and because kinetics analysis of cotyledon unfolding showed that the response is displayed after the third daily pulse (Figure 1B).

We identified 208 genes showing significant effects of treatments ($P < 0.05$, $q < 0.10$), significant effects of red light ($P < 0.05$), and significant effects of phyA ($P < 0.05$) (Supplemental Table 1). These genes were grouped in four categories depending on whether their expression was promoted or inhibited by red light and whether this response was reduced or enhanced in the *phyA* mutant: hypo-promoted by red light in *phyA*, hyper-promoted by red light in *phyA*, hypo-inhibited by red light in *phyA*, and hyper-inhibited by red light in *phyA* (Figure 1C). The genes hyper-responsive to red light in the *phyA* mutant significantly

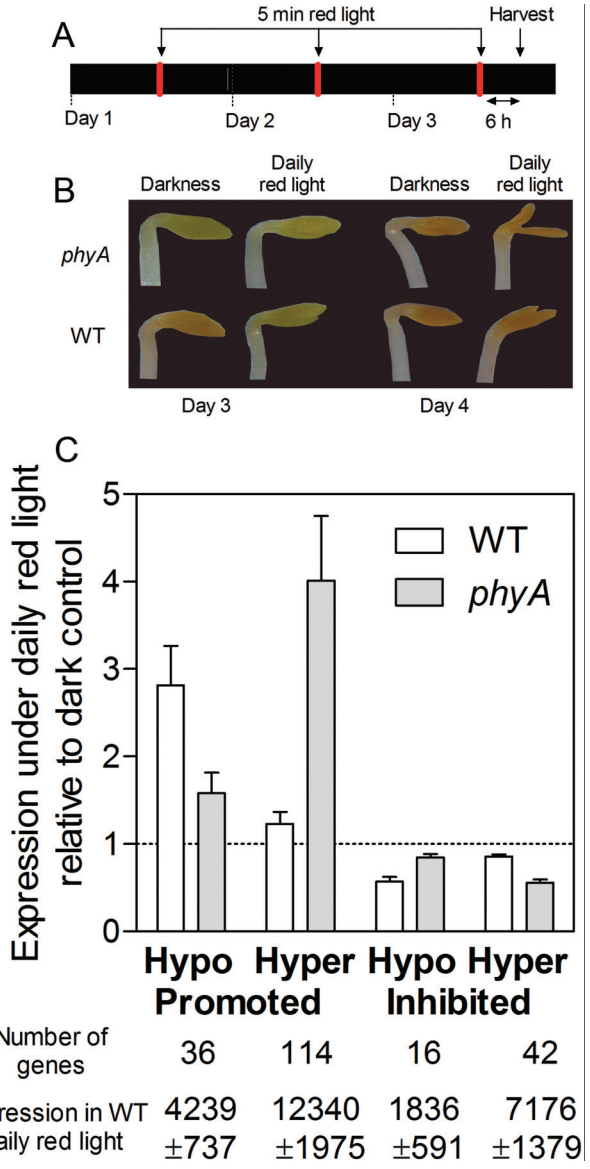

Figure 1 Genomic Analysis of the Effect of a Daily Red Light Pulse Shows Enhanced Responses in the *phyA* Mutant.

(A) Experimental protocol: 1-day-old etiolated, wild-type (WT), and *phyA* mutant seedlings were exposed to three daily pulses (5 min) of red light and harvested 6 h after the last pulse. Dark controls were harvested simultaneously.

(B) Detail of the cotyledon of representative seedlings of the WT and the *phyA* mutant (Landsberg *erecta*) grown for 3 or 4 d either in full darkness or under daily pulses of red light.

(C) Average expression patterns of the four groups defined by the effect of red light (promotion or inhibition) and the impact of the *phyA* mutation (reduced or enhanced response to red light) (Supplemental Table 1). The expression of each gene was normalized to the expression in darkness and averaged for each group. The number of genes and the average expression in red light-treated seedlings (not normalized to darkness) are also indicated for each group.

outnumbered those showing reduced response to red light in the *phyA* mutant. This confirms that the selected protocol favored the negative effects of phyA on the responses to red light. The effects were large, as, for instance, hyper-promoted genes showed an average four-fold response to red light in *phyA* compared to a 1.2-fold response in the WT (Figure 1C).

To investigate the generalities of the genes significantly affected by phyA, we calculated the average expression levels for WT seedlings treated with red light in each category. For a given gene, the output of microarray experiments depends on the actual expression levels and the sensitivity of the probes for this gene but, when groups of genes are considered, differences in probe sensitivity should tend to cancel each other. In the WT, the genes hyper-responsive to red light in the *phyA* mutant tend to have higher expression levels than the genes hypo-responsive to red light in the *phyA* mutant (Figure 1C). This was observed, despite the fact that the genes that are hyper-responsive to red light in *phyA* tend to show poor response to red light in the WT compared to the hypo-responsive genes (see red light/darkness ratios of the WT in Figure 1C).

To investigate the functional significance of the negative regulation of light responses by phyA, we identified over-represented gene ontology terms. Thylakoid- ($P = 7.1 \times 10^{-24}$), photosynthesis- ($P = 1.6 \times 10^{-13}$), and porphyrin biosynthesis- ($P = 1.2 \times 10^{-6}$) related genes were strongly over-represented among the genes hyper-promoted by red light in *phyA*. Chloroplast-membrane- ($P = 2.4 \times 10^{-3}$), chloroplast-stroma- ($P = 4.5 \times 10^{-3}$), and chloroplast organization and biogenesis- ($P = 1.3 \times 10^{-3}$) related genes showed weak overrepresentation among the genes hypo-promoted by red light in the *phyA* mutant.

To further characterize the genes showing hypersensitivity to red light in the *phyA* mutant, we analyzed their expression in publicly available microarray data. We observed that the genes affected by *phyA* in our conditions (both hypo- and hyper-responsive genes, 6 h after a brief pulse of red light) tend to show little or no early response to red light (1 h) and no effects of *phyA* (Tepperman et al., 2006; Supplemental Figure 1). Both genes showing hypo- and hyper-promotion by red light in *phyA* showed similar levels of promotion under continuous far-red light (WT, 6 h) (Staneloni et al., 2009; Supplemental Figure 1). Those inhibited by red light in a phyA-dependent manner showed poor response to continuous far-red light (Supplemental Figure 1).

The Hyper-Response to Red Light of the *phyA* Mutant Requires PIF1

To investigate the mechanisms causing the enhanced response to red light in the *phyA* mutant, we searched for

transcription factor genes showing expression correlation in publicly available microarray data with the group of genes hyper-promoted by red light in *phyA*. The top 40 list included nine basic helix–loop–helix transcription factor genes, which was the most abundant class of transcription factors. In turn, the latter included *PIF4*, *PIF5*, and *PIF1* (Supplemental Table 2). Since PIF1 physically interacts with phyA and phyA induces its phosphorylation and degradation (Shen et al., 2005, 2008), we selected PIF1 for further studies. The correlation between PIF1 and hyperactive genes was weak but positive. Therefore, our hypothesis was that phyA could reduce the response to red light by causing the degradation of PIF1, which would promote the response to red light in these hyper-responsive genes.

A corollary of the above hypothesis is that the response to red light should be enhanced in the absence of phyA but in the presence of PIF1; that is, the hyper-response to red light observed in *phyA* compared to the WT should be absent in *phyA pif1* compared to *pif1*. To test this prediction, we selected seven genes of the list of those hyper-promoted by red light in *phyA* for analysis in the WT and the *phyA*, *pif1*, and *phyA pif1* mutants in the same protocol described in Figure 1A. The criteria for gene selection were based on the magnitude of the hyper-response to red light in *phyA*, the magnitude of the basal expression, and the presence of the PIF1-binding site (Moon et al., 2008) in their promoters. For the statistical analysis, expression data were fitted to the model: $y = a + bx_1 + cx_2$, where a represents the expression in darkness; b the basal effect of red light ($x_1 = 0$ for dark controls and $x_1 = 1$ for red light-treated samples); and c the hyper-response ($x_2 = 1$ for the *phyA* mutant exposed to red light and $x_2 = 0$ for all the other conditions because the hyper-response would require the absence of phyA and the presence of PIF1).

Despite the fact that the genes had been selected on the basis of their hyper-response to red light in the *phyA* mutant compared to the WT in Landsberg *erecta*, the seven genes showed a stronger promotion by red light in the *phyA* mutant than in the WT in real-time RT–PCR experiments in the Columbia background (Figure 2). Five of the seven genes showed a significant contribution of *c*, confirming that the hyper-response to red light in *phyA* requires PIF1 (Figure 2). The other two genes (*PIF4* and *PSBQ2*) showed some effects of the mutations (*pif1*, *phyA*) in darkness. In *phyA*, the expression of *PIF4* was slightly lower than in the WT—a response also observed in publicly available data (Tepperman et al., 2006). Both *PIF4* and *PSBQ2* showed enhanced dark expression in the *pif1* background. These effects in darkness could have reduced the significance of the term including *c*. In accordance with this interpretation, when the model is applied to *PIF4* and *PSBQ2* expression data normalized to the dark controls of each genotype, the term including *c* becomes highly significant (Figure 2).

In subsequent experiments, the seedlings were exposed to the same protocol and the angle between the cotyledons was measured 24h after the last daily red light pulse. The response to daily red light is reduced in Columbia (Figure 2) compared to Landsberg *erecta* (Figure 1B). The term *c* was also highly significant for this physiological variable (Figure 2), confirming that the hyper-promotion of de-etiolation by red light in *phyA* requires PIF1. The response to daily red light observed in the *phyA* mutant is mediated by phyB and it is therefore absent in the *phyA phyB* double mutant (cotyledon angle under daily red light = 0.1±0.1 degrees, mean and SE, eight replicate boxes, not significantly different from 0).

Enhanced Stability of PIF1 in *phyA* under Daily Pulses of Red Light

The hypothesis proposed here is that phyA reduces the response to red light by causing the degradation of PIF1, which promotes the response to red light of hyper-responsive genes. Another corollary of this hypothesis is that PIF1 should be more abundant under daily pulses of red light in the *phyA* mutant—that is, under the conditions where the *phyA* mutant is shown to be hyperactive in a PIF1-dependent manner (Figure 2). Figure 3 shows that the *phyA* mutant does retain more PIF1 after daily red light pulses, which confirms and extends previous observations using a single red light pulse to dark-grown seedlings (Shen et al., 2005, 2008).

PIF1 Binds *In Vivo* to the Promoters of Genes with Response to Red Light Enhanced by PIF1

To gain insight into the mechanisms involved in the positive effects of PIF1 on de-etiolation, we selected three genes of the list included in Figure 2 (*TROL*, *PIF4*, and *PBS27*) to investigate whether these genes with response to red light enhanced by PIF1 are directly regulated by PIF1. We used a line where the *pif1* mutation is complemented by the TAP–PIF1 construct expressed from the native PIF1 promoter construct (Bu et al., 2011). We immunoprecipitated DNA–protein complexes by using antibody to MYC tag and calculated the ratio between the output of real-time PCR obtained with primers specific for the G-box or to control regions of the promoters. The data in Figure 4 indicate that *TROL*, *PIF4*, and *PBS27* are direct targets of PIF1.

PIF1 Mutated at the Phytochrome-Binding Domains Reduces Selected Responses to Red Light

To test whether the proposed positive contribution of PIF1 to gene expression responses to red light requires

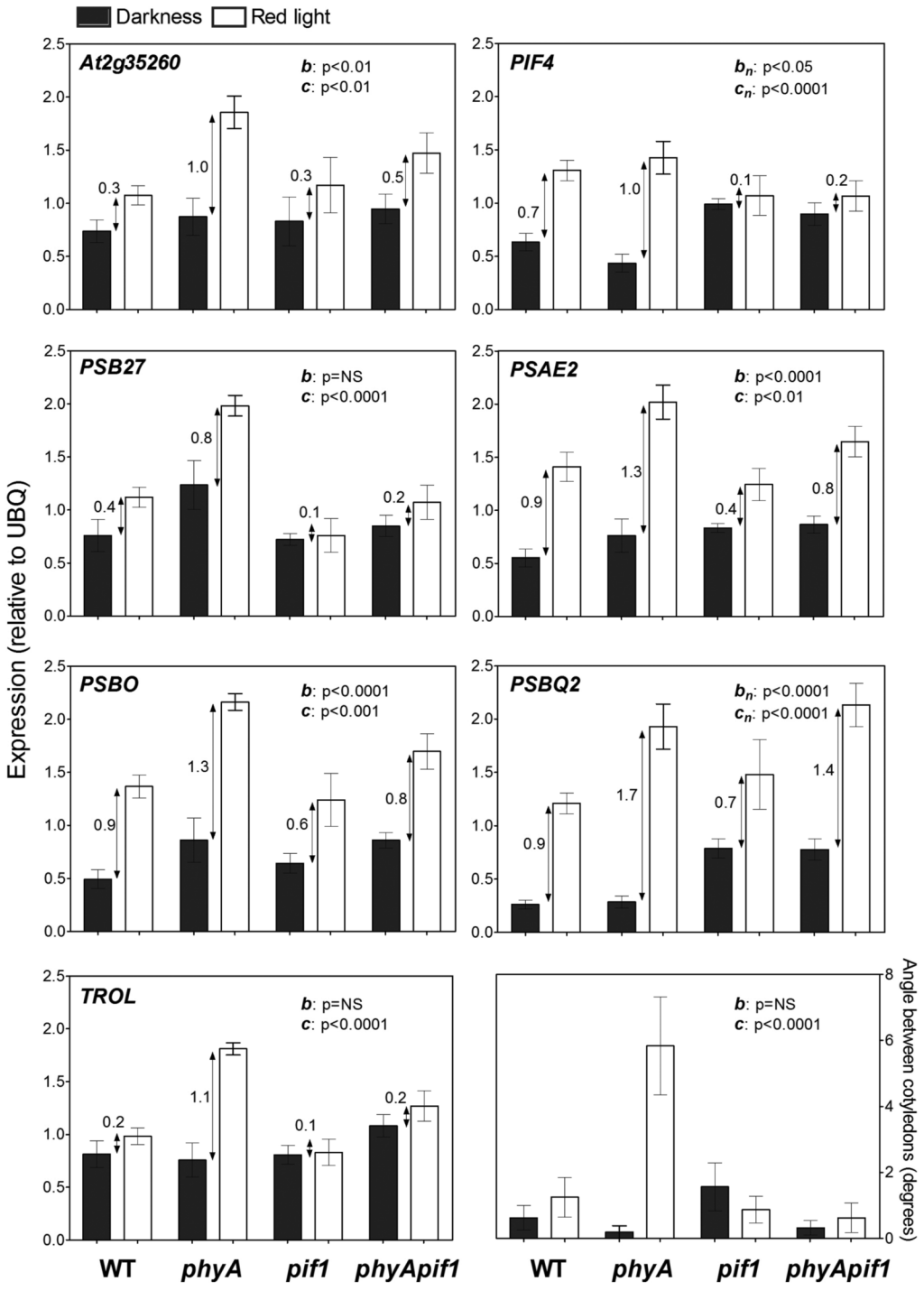

Figure 2 The Enhanced Response to Daily Red Light Observed in the *phyA* Mutant Requires PIF1.

Seedlings of the wild-type and of the *phyA*, *pif1* and *phyA pif1* mutants were exposed to daily red light and either harvested 6 h after the last pulse (as in Figure 1A) for the analysis of gene expression or 24 h after the last pulse to measure cotyledon angle (the quantitative differences in angle with respect to Figure 1B reflect the use of different accessions). Data were fitted to the model: $y = a + bx_1 + cx_2$, where *a* represents the expression in darkness; *b* the basal effect of red light ($x_1 = 0$ for dark controls and $x_1 = 1$ for red light-treated samples); and *c* the hyper-response ($x_2 = 1$ for the *phyA* mutant exposed to red light and $x_2 = 0$ for all the other conditions). The significance of the terms *b* and *c* is indicated. The term *c* was not significant ($P > 0.05$) for the expression of *PIF4* and *PSBQ2* but, when the expression of each genotype was normalized to the values observed in darkness, the term becomes significant (see b_N and c_N for these genes). Data are means and SE of three (real-time RT–PCR expression data) or 26–52 (angle between cotyledons) independent biological replicates.

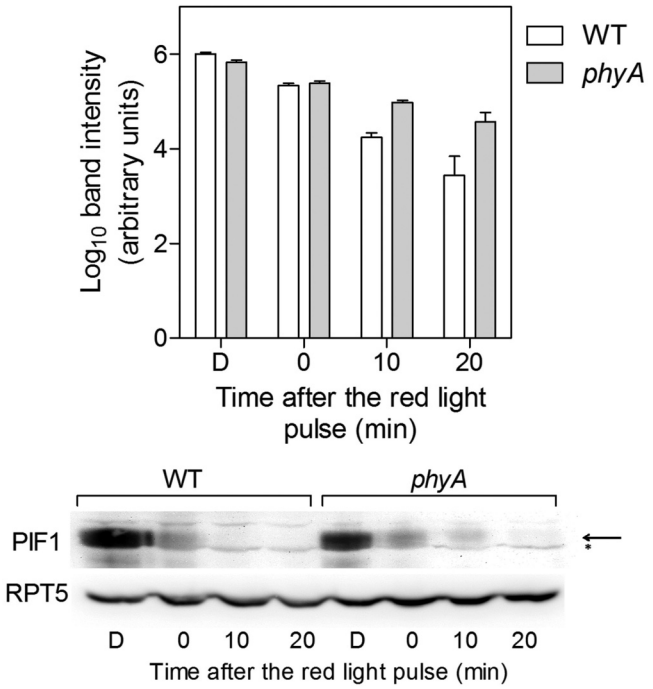

Figure 3 Increased Abundance of PIF1 in *phyA* Mutant Seedlings Exposed to Daily Pulses of Red Light.

Levels of PIF proteins were measured with a native antibody in 4-day-old dark-grown seedlings either kept in the dark (D) or exposed to a daily pulse of red light (5 min, 3000 μmol m^{-2}) followed by incubation in the dark for 10 and 20 min. The arrow points to PIF1 protein and the asterisk indicates a cross-reacting band. Data are mean ± SE of two biological replicates and a representative protein blot is included.

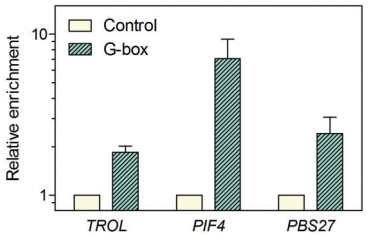

Figure 4 PIF1 Directly Binds Genes with Response to Red Light Enhanced by PIF1.

The ChIP assay was performed on 3-day-old dark-grown seedlings expressing the TAP–PIF1 fusion protein from the native *PIF1* promoter. Relative enrichment represents the ratio between real-time PCR products obtained with primers specific to the region containing the G-box element and with primer specific to control regions in *TROL*, *PIF4*, and *PBS27* genes. Data are means ± SE of three to six biological replicates.

phytochrome binding, the *pif1*- mutant was transformed with either the WT *PIF1* gene or the *PIF1* gene mutated in phyA and phyB binding sites (APA and APB), both under the control of the native *PIF1* promoter (Figure 5A). As expected, the mutation increased PIF1 stability under red light, without affecting PIF1 abundance in darkness (Figure 5A).

We repeated the experiment described in Figure 2 but with these transgenic lines. Since the lines with mutated PIF1 have elevated levels of PIF1, we expected enhanced responses to red light compared to the line bearing WT PIF1. None of the seven genes or cotyledon unfolding showed a stronger response to red light in the transgenics with impaired PIF1 ability to bind phytochrome (Figure 5B). On the contrary, some genes actually showed a response to red light that was inversely related to PIF1 levels. For the statistical treatment, data were fitted to the model: $y = a + bx_1 + cx_2$, where a represents the expression in darkness; b the basal effect of red light; and c the interaction between red light and PIF1 levels (the average between 0 and 30 min in Figure 5A). The coefficient c was negative and statistically significant for *PIF4*, *PSB27*, *PSBO*, and *TROL* (Figure 5B) indicating that, for these genes, enhanced PIF1 levels reduced expression in response to red light. Note that, for these genes, the TAP–PIF1 24–6 line showed the lowest levels of PIF1 (Figure 5A) and the strongest responses to red light (Figure 5B), and the opposite was true for the TAP–PIF1 14–1 line. None of the genes showed a positive slope. We conclude that the enhanced gene expression responses to red light require PIF1 binding by phytochromes.

Greening Hyper-Sensitivity to Light in *phyA* Requires PIF1

The light protocol used in all previous experiments (daily pulse with red light) was designed to focus on the enhanced responses to red light. The GO terms thylakoid, photosynthesis, and porphyrin biosynthesis were over-represented among the genes showing hyper-promotion by red light in the *phyA* mutant, suggesting that the interaction between phyA and PIF1 could also be involved in the control of greening, giving the opportunity to investigate whether the genetic interaction between phyA and PIF1 also works under stronger light inputs. We measured chlorophyll levels in 3-day-old seedlings de-etiolated for 24 h under different irradiances of orange light (Figure 6A). The WT showed a biphasic response, with an optimum between 25 and 50 μmol m^{-2} s^{-1} (Figure 6B). While light is necessary for chlorophyll synthesis, high irradiances are known to reduce chlorophyll levels (Rossini et al., 2006). In the *phyA* mutant, maximum chlorophyll levels were attained at lower irradiances (5 μmol m^{-2} s^{-1}; Figure 5B), indicating at least a five-fold higher sensitivity than in the WT. As expected for 3-day-old seedlings (Huq et al., 2004), the *pif1* mutant had little difference with the WT (Figure 6B and 6C). The *phyA*

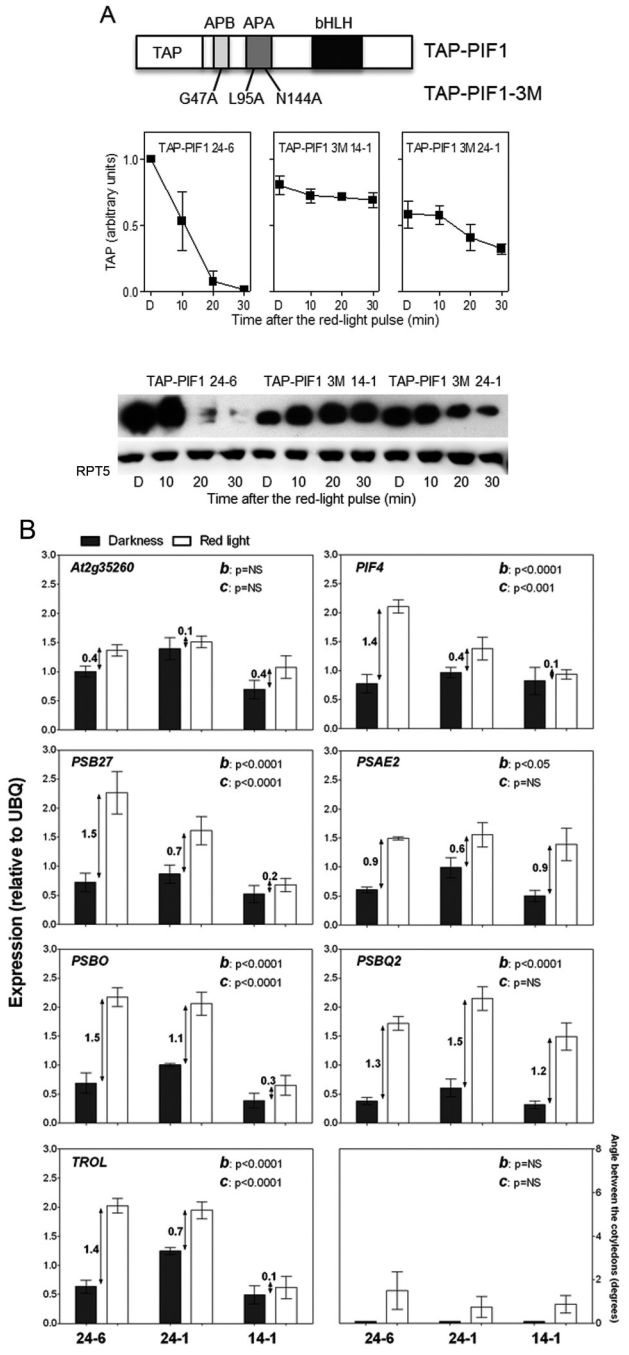

Figure 5 PIF1 Mutated at the Phytochrome-Binding Domains Reduces Selected Responses to Red Light.

(A) Lines expressing either the wild-type (WT) PIF1 (24–6) or the mutant (3M) PIF1 (14–1 and 24–1). A scheme of the constructs is shown at the top. WT and mutant genes were expressed under the control of the native *PIF1* promoter in the *pif1* background. Levels of PIF proteins in 4-day-old dark-grown seedlings either kept in the dark (D) or exposed to a pulse of red light followed by incubation in the dark for 10, 20, and 30 min are shown as mean ± SE of three biological replicates and a representative protein blot is included.

pif1 double mutant showed reduced greening and optimum irradiance values close to those observed for the WT (Figure 6C). This indicates that the *phyA* mutation increases the sensitivity to red light in the presence of PIF1 but not in its absence, resembling the pattern observed for the magnitude of gene expression responses to red light (Figure 2).

Contrasting Effects of PIF1 on phyA-Dependent and -Independent Photomorphogenesis

The results presented in Figures 2 and 5 indicate a positive role of PIF1 on photomorphogenesis, which on the surface appears to contradict the negative effect described in the literature (Oh et al., 2004; Shen et al., 2005, 2008). To explore this issue in further detail, we used 3-min red and far-red light pulses applied hourly instead of the daily 5-min pulses used in the above experiments. We changed the protocol to extend the list of physiological outputs to be evaluated as hourly red or far-red light pulses are effective to reduce final hypocotyl length, enhance hypocotyl angle to the vertical axes (reduce gravitropism), and enhance cotyledon unfolding, whilst daily red only affects cotyledon unfolding and daily far-red has no detectable morphological responses. The *pif1* mutation had no effects on hypocotyl length in dark-grown seedlings (mm, WT = 11.8 ± 0.1; *pif1-1* = 12.0 ± 0.1; *pif1-2* = 12.2 ± 0.2, data are means ± SE of 30–59 replicate boxes) (Shin et al., 2009). The *pif1* mutation had a significant effect on cotyledon unfolding in darkness but these effects were very small when compared to those induced by hourly red or even far-red light pulses (degrees, WT = 0.2 ± 0.1; *pif1-1* = 0.8 ± 0.2, $P < 0.01$; *pif1-2* = 1.2 ± 0.3, $P < 0.001$; data are means ± SE of 30–59 replicate boxes and the significance of Bonferroni Multiple Comparison Tests with the WT is indicated). The angle of the hypocotyl to the vertical axes was significantly increased by the *pif1* mutation (Oh et al., 2004; Shen et al., 2005, 2009) and this effect was in the range of the response to hourly far-red light (Figure 7C, black bars). These effects in darkness are consistent with those reported in the literature.

(B) Seedlings of the 24–6, 14–1, and 24–1 lines were exposed to daily red light and harvested either 6 h after the last pulse (as in Figure 1A) for the analysis of gene expression or 24 h after the last pulse to measure cotyledon angle. Data were fitted to the model: $y = a + bx_1 + cx_2$, where a represents the expression in darkness; b the basal effect of red light ($x_1 = 0$ for dark controls and $x_1 = 1$ for red light-treated samples); and c the interaction between red light and PIF1 levels ($x_2 = 0$ for dark controls and $x_2 = 0.41$, 0.47, and 0.76 for red light-treated seedlings of the 24–6, 14–1, and 24–1 lines, respectively). PIF1 levels are the average between 0 and 30 min in (A). The significance of the terms b and c is indicated. Data are means and SE of three (real-time RT–PCR expression data) or six to eight (angle between cotyledons) biological replicates.

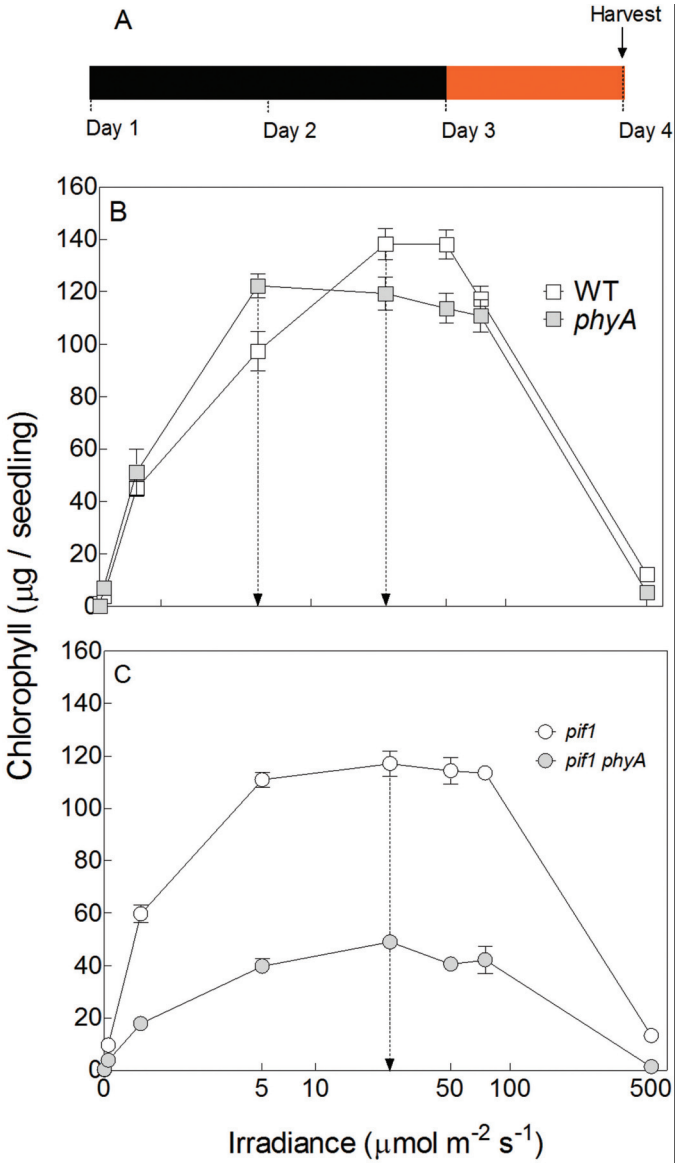

Figure 6 Enhanced Sensitivity of Greening in the *phyA* Mutant in the Presence of PIF1.

(A) Experimental protocol: 3-day-old etiolated, wild-type, and *phyA*, *pif1*, and *phyA pif1* mutant seedlings were exposed to 24 h of orange light before harvest.

(B, C) Chlorophyll levels. Data are means ± SE of five replicate boxes. The irradiance of maximum chlorophyll levels is indicated.

Under hourly red light, hypocotyl growth inhibition relative to dark controls (we use inhibition to aid the comparison of the shape of the different physiological outputs) and cotyledon unfolding were enhanced by the *phyA* mutation (approximately 1.7-fold enhanced response), but the hypersensitivity was eliminated by the *pif1* mutation (Figure 6A and 6B, white bars). Under hourly far-red light, inhibition of hypocotyl growth and cotyledon unfolding were strictly phyA-dependent (Casal and Boccalandro, 1995; Yanovsky et al., 1997). In contrast to the phyA-independent response under red light, the phyA-dependent response to hourly far-red light was enhanced by the *pif1* mutation (Figure 7A and 7B, gray bars). The response of the angle of the hypocotyl to the vertical axes to red light was not obviously enhanced by the *phyA* mutation and that to far-red was unaffected by the *pif1* mutation (Figure 7C). The *phyA phyB* double mutant shows no obvious morphological responses to hourly red or far-red light (Mazzella et al., 1997; Cerdán et al., 1999), indicating that the response to red light observed in the *phyA* mutant and dependent on PIF1 is mediated by phyB. In accordance with this notion, the effect of PIF1 (i.e. the difference between *phyA* and *phyA pif1*) was reduced by far-red light added to the red light pulses (Supplemental Figure 2).

Increased PIF1 Abundance Enhances Photomorphogenesis

We also analyzed whether phytochrome binding was necessary for the enhanced growth responses to hourly light pulses. Compared to the line bearing WT PIF1, those expressing PIF1 mutated at APA and APB (Figure 5A) showed enhanced hypocotyl growth and cotyledon angle responses to hourly red light (Figure 8). The lines with higher PIF1 levels (14–1, 24–1) demonstrate a positive effect of PIF1 on photomorphogenesis under hourly red light, which does not require the presence of the *phyA* mutation. Conversely, the lines with higher PIF1 levels showed reduced the response to hourly pulses of far-red light (Figure 8). PIF1 overexpression lines have reduced hypocotyl growth responses not only under far-red light, but also under red light (Oh et al., 2004), but these lines where *PIF1* expression is under the control of a constitutive promoter are expected to have higher PIF1 levels even in darkness, whilst differences in darkness between *pPIF1:TAP–PIF1* and *pPIF1:TAP–PIF1-3M* lines used here were small (Figure 5A).

DISCUSSION

Under prolonged red light (Mazzella et al., 1997; Cerdán et al., 1999; Franklin et al., 2007; Torres-Galea et al., 2013) or sunlight (Mazzella and Casal, 2001; Casal et al., 2014), phyA mediates de-etiolation but inhibits phyB-mediated de-etiolation. The negative branch of phyA action is often stronger than the positive one and, therefore, the *phyA* mutant is hyper-photomorphogenic. The analysis of a network based on the genes showing hyper-promotion of expression by red light in the *phyA* mutant compared to the WT pointed to PIF1 as a player in the negative effect of phyA on the responses to red light. The analysis of the *phyA pif1* double mutant confirmed that the hyper-photomorphogenic phenotype of *phyA* requires PIF1 (Figures 2 and 6–9).

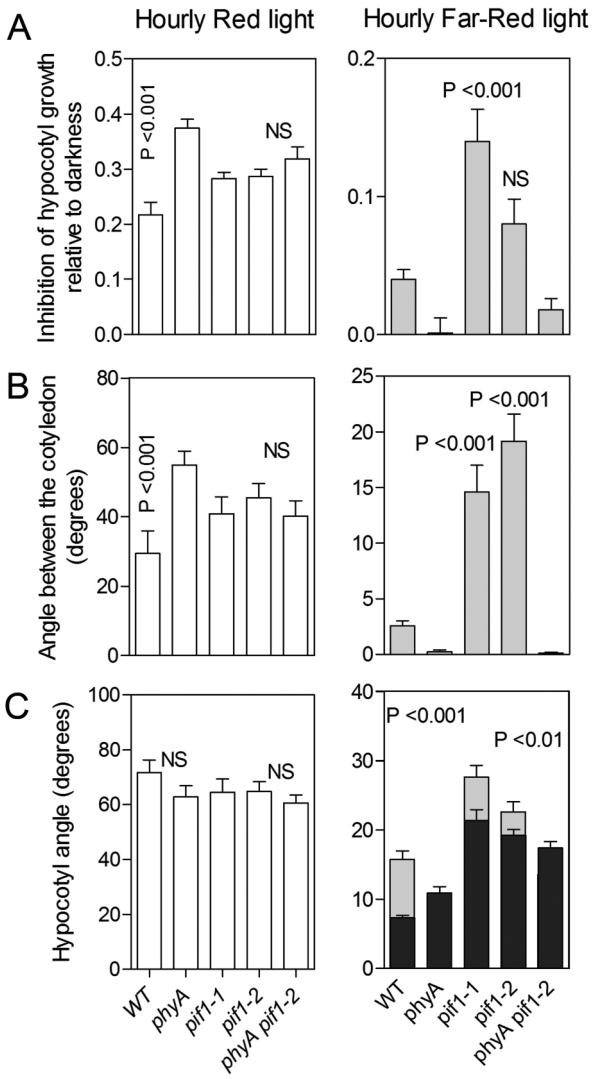

Figure 7 Contrasting Effects of PIF1 on phyA-Dependent and -Independent Photomorphogenesis.

One-day-old seedlings of the wild-type (WT) and of the *phyA*, *pif1*, and *phyA pif1* mutants were exposed to hourly red or far-red light pulses for 3 d before measurements of hypocotyl length **(A)**, angle between the cotyledons **(B)**, or hypocotyl angle **(C)**. Dark controls were included to calculate the inhibition of hypocotyl growth ([length in darkness – length under light pulse]/length in darkness) (A). Cotyledon angle in darkness was small (less than 2 degrees) and hypocotyl angle in darkness is indicated by the black bars (C). Data are means and SE of 10–30 replicate boxes. Data were analyzed by ANOVA followed by Bonferroni's Multiple Comparison Test between WT and *phyA* and between *pif1 phyA* and *phyA* under hourly red light and between WT and each *pif1* mutant allele under hourly far-red light. The significance is indicated. NS, not significant.

In darkness, PIF1 negatively regulates the biosynthesis of protochlorophyllide (Huq et al., 2004) and the accumulation of carotenoids (Toledo-Ortiz et al., 2010a) by reducing the expression of a discrete set of genes, including *HEMA1*, *GENOMES UNCOUPLED 4* (*GUN4*), *CONDITIONAL CHLORINA* (*CHLH*), protochlorophyllide oxidoreductase, ferrochelatase and heme oxygenase (Moon et al., 2008; Stephenson et al., 2009), and the gene encoding phytoene synthase (Toledo-Ortiz et al., 2010a), respectively. Some of these genes are direct targets of PIF1 (Moon et al., 2008; Toledo-Ortiz et al., 2010a). In darkness, PIF1 also promotes hypocotyl growth and hypocotyl negative gravitropism, inhibits cotyledon unfolding, and controls gene expression with different degrees of redundancy with other PIFs (Oh et al., 2004; Shen et al., 2005; Leivar et al., 2008; Shin et al., 2009; Leivar et al., 2012; Zhang et al., 2013). PIF1 is a repressor of photomorphogenesis in darkness and light activation of phyA and phyB induces its degradation in the proteasome (Shen et al., 2005, 2008) and the initiation of photomorphogenesis (Leivar et al., 2008; Shin et al., 2009; Leivar et al., 2012) (Figure 9). The short-hypocotyl phenotype of *pif1* is enhanced by far-red light in a phyA-dependent manner (Oh et al., 2004) (Figure 7). In contrast to this role as negative regulator of photomorphogenesis, here we report a positive role of PIF1 in photomorphogenesis under red light (Figure 9), including hypocotyl growth, cotyledon unfolding, greening, and gene expression responses to red light (Figures 2 and 6–8).

The positive effect of PIF1 on light responses was observed in the *phyA* mutant background (Figure 7) at least in part because phyA induces PIF1 degradation and PIF1 accumulates in *phyA* (Shen et al., 2008) (Figures 3 and 9). In fact, seedlings expressing a light-stable form of PIF1 showed enhanced inhibition of hypocotyl growth and promotion of cotyledon unfolding by red light without the *phyA* mutation (Figure 8). phyA could also release PIF1 from its DNA targets as reported for phyB (Park et al., 2012).

The enhanced de-etiolation under red light could result from the transcriptional activity of PIF1. In favor of this interpretation, we observed binding of PIF1 to the promoter of genes with expression responses to red light enhanced by PIF1 in the absence of phyA (Figure 4).

The positive effect of PIF1 on de-etiolation required phytochrome binding for some light responses (short-term gene expression responses to red light; Figure 5) but not for others (long-term morphological responses to red light; Figure 8).The requirement of phytochrome binding might indicate that PIF1-enhanced transport of phyB to the nucleus (Pfeiffer et al., 2012) could contribute to its positive effect on de-etiolation. PIF1 could also recruit phyB to gene promoters as PIF3 does with phyA (Chen et al., 2012). Alternatively, partial phytochrome-mediated degradation of PIF1 might be needed as in the cases called 'activation by destruction' (Lipford et al., 2005; Iñigo et al., 2012).

PIF3 is involved in the repression of photomorphogenesis in darkness (Leivar et al., 2008; Shin et al., 2009) and under red light (Bauer et al., 2004; Monte et al., 2004), but

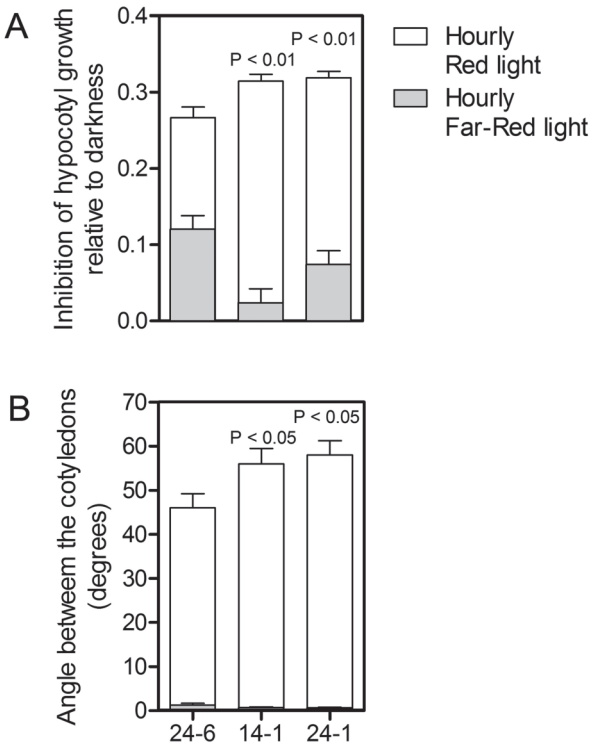

Figure 8 PIF1 Mutated at the Phytochrome-Binding Domains Enhances Hypocotyl Growth- and Cotyledon Angle-Responses to Red Light.

One-day-old seedlings were exposed to hourly red or far-red light pulses for 3 d before measurements of hypocotyl length **(A)** and angle between the cotyledons **(B)**. Data are means and SE of 10–26 replicate boxes. Data were analyzed by ANOVA followed by Bonferroni's Multiple Comparison Tests involving each line bearing mutant *PIF1* and the control line bearing WT *PIF1* (the significance is indicated).

PIF3 is also required for the initial phases of light-induced chloroplast development and greening via the regulation of a subset of rapidly light-induced nuclear genes encoding plastid and photosynthesis-related components under red light (Monte et al., 2004; Zhang et al., 2013) and for the phyA-mediated induction of anthocyanin biosynthesis genes under far-red light (Shin et al., 2007). PIF4 is involved in the repression of photomorphogenesis in darkness (Leivar et al., 2008; Shin et al., 2009) and under red light (Huq and Quail, 2002), but PIF4 works in the same direction as phyB in the control of stomatal development in response to red light (Casson et al., 2009) and in the repression of C-repeat binding factor (CBF) pathway and freezing tolerance under long days (Lee and Thomashow, 2012). Therefore, PIF3 and PIF4 appear to act positively on selected light responses, as reported here for PIF1 effects on hypocotyl growth, cotyledon unfolding, greening sensitivity, and gene expression in response to red light.

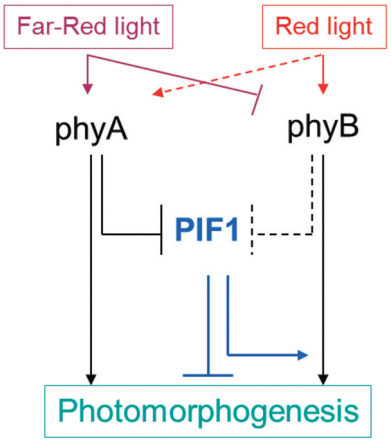

Figure 9 Repression and Promotion of Photomorphogenesis by PIF1.

PIF1 represses photomorphogenesis in darkness. Light activation of phyA (and secondarily phyB) antagonizes PIF1 as part of the processes that initiate de-etiolation. In addition, PIF1 has a positive effect on phyB-mediated photomorphogenesis, which becomes more evident in the *phyA* mutant background, where PIF1 becomes more stable.

De-etiolation is initiated by the transition between the full darkness of the soil and daily light cycles. Therefore, changes in other variables of the light environment, such as the red/far-red ratio (affected by canopy shade), have to be compensated for. The positive action of phyA on photomorphogenesis serves this purpose because it shows maximal activity under far-red light, complementing the spectral dependence of phyB (which is maximal under red light). The phyA–PIF1 module would also provide de-etiolation homeostasis against differences in the red/far-red ratio immediately after emergence from the soil. In fact, PIF1 enhances photomorphogenesis under red light and reduces photomorphogenesis under far-red light (Figures 7 and 8); that is, PIF1 enhances the difference between red and far-red light-rich conditions. Therefore, by inducing PIF1 degradation, phyA would further reduce the difference between red and far-red light-rich conditions early during de-etiolation.

METHODS

Plant Material and Growth Conditions

The WT Landsberg *erecta* and the *phyA-201* mutant (Nagatani et al., 1993) were used for microarray experiments. The WT Columbia, and the mutants *phyA-211* (Reed et al., 1994), *pif1-2* (in some cases also *pif1-1*) (Huq et al., 2004), and *pif1-2 phyA-211* (Castillon et al., 2009) were used in the rest of the experiments. Seeds of *Arabidopsis thaliana* were sown on 0.8% agar in clear plastic boxes and stratified for 4 d at 4°C in darkness. Stratified seeds were transferred to 22°C,

irradiated with red light (3 h, 5 μmol m^{-2} s^{-1}) to induce germination and incubated in darkness either 1 d before transfer to hourly (3 min) red or far-red light pulses or daily red light pulses (5 min) for 3 d (Cerdán et al., 1999). Alternatively, seedlings were grown in darkness for 3 d and then exposed 24 h to different irradiances of orange light provided by low-pressure sodium lamps (Philips SOX 180W) wrapped in one yellow (Lee Filters, 101) and one orange (Lee Filters, 105) filter.

Analysis of the Transcriptome

Total RNA was extracted with the RNEasy Plant mini kit (Qiagen, www.qiagen.com) following the manufacturer's protocols. cDNA and cRNA synthesis and hybridization to ATH1 Affymetrix Arabidopsis Gene Chips were performed in accordance with Affymetrix instructions. For the statistical analysis, we used three groups: one group containing three biological replicates from WT seedlings under daily red light, a second group containing three biological replicates for the *phyA* mutant under daily red light, and a third group containing one sample for the WT in darkness and one sample for the *phyA* mutant in darkness. The purpose of this procedure was to make the selection more stringent and focused on the responses to red light and not on pre-existing differences between *phyA* and WT in darkness. The differences between *phyA* and WT in darkness increased the error estimates (because both genotypes composed one treatment) and the threshold necessary to overcome the statistical cut-off. We selected the genes showing significant effects of treatment ($p < 0.05$, $q < 0.10$; Storey and Tibshirani, 2003), significant effects of red light ($p < 0.05$), and significant effects of the *phyA* mutation ($p < 0.05$). Over-represented gene ontology terms were investigated by using the ATCOESIS homepage (Vandepoele et al., 2009). The list of transcription factors showing co-expression with the genes hyper-promoted by daily red light in the *phyA* mutant compared to the WT was obtained using the ATTED-II webpage (Obayashi et al., 2011).

Real-Time RT–PCR

RNA was extracted by using Trizol (Invitrogen). RNA concentration was quantified using an Ultrospec 2100 Pro Spectrophotometer (Amersham Biosciencies). Two micrograms total RNA were transcribed into cDNA with Superscript III reverse transcriptase (Invitrogen) according to the protocol provided by manufacturer using a (dT)15 primer. We included controls for genomic DNA contamination by following the same procedures but omitting the addition of reverse transcriptase. Real-time PCR was carried out using 2 μl of three-fold diluted solution of cDNA in a total reaction volume of 10 μl (5 μl FastStart SYBR Green PCR MIX–Roche/2 μl sterilized water/1 μl 5 mM primers mix). Each cDNA sample was analyzed twice in a 7500 Real Time PCR System (Applied Biosystems). The 7500 System SDS software (Applied Biosystems) was used for calculations. The primers are given in Supplemental Table 3.

Plant Measurements

The length of the hypocotyl was measured with a ruler and the average of the 10 tallest seedlings of each box (out of 15 seeds sown per box) was used as one replicate. Data are presented as inhibition of hypocotyl growth relative to the dark controls ([length in darkness – length under light]/length in darkness) (Staneloni et al., 2009). The angle between the cotyledons was measured in the same 10 seedlings with a protractor (Staneloni et al., 2009). For hypocotyl angle experiments, the seeds were sown forming a row on the agar and the agar was rotated to the vertical position after the induction of seed germination. As a result of this, the seedlings grew attached to the agar surface and their angle was measured with a protractor (Staneloni et al., 2009). For chlorophyll measurements (Moran, 1982), seedlings were harvested in cold Dimethylformamide (1 ml, 15 plants ml^{-1}) and incubated for 3 d in darkness at –20°C.

Construction of Plasmids and Generation of Transgenic Plants

The pENTRY clone of PIF1 and the WT TAP–PIF1 construct (*pPIF1:TAP–PIF1*) are as described (Bu et al., 2011). The APA and APB mutations in full-length PIF1 (Shen et al., 2008) were introduced using a site-directed mutagenesis kit in pENTRY vector background (Stratagene, La Jolla, CA). The resulting pENTRY clone of PIF1 was recombined with pNTAPa (Rubio et al., 2005) to produce 35S:TAP–PIF1-3M. The cloning of a 1.6-kb *PIF1* promoter fragment into pPZP121 vector was previously described (Bu et al., 2011). A 3.5-kb fragment containing the TAP–PIF1-3M from the 35S:TAP–PIF1-3M construct was cloned into the pPZP121–pPIF1 to generate *pPIF1:TAP–PIF1-3M* in the pPZP121 background. The final construct was verified by sequencing, transformed into Agrobacterium strain GV3101 by electroporation, and then transformed into the *pif1-2* background using the floral dip method. Transgenic plants were selected on gentamycin and homozygous lines were selected from single insert lines.

Protein Extraction and Western Blotting

Protein extraction and Western blotting were performed essentially as described (Shen et al., 2008; Bu et al., 2011). Briefly, seedlings were grown in the dark for 4 d and then either kept in darkness or exposed to a pulse of red light (3000 μmol m^{-2}) followed by incubation in the dark for various times as indicated in the figure. Total proteins were extracted in boiling denaturing buffer (100 mM

MOPS, pH 7.6, 5% SDS, 10% Glycerol, 4 mM EDTA, 40 mM β-mercaptoethanol, 1X protease inhibitor cocktail (F. Hoffmann-La Roche Ltd, Basel, Switzerland)) (1:3, w/v ratio). PMSF (2 mM) was also added during extraction. Total proteins were separated on a 6% SDS–PAGE gel, blotted onto PVDF membrane, and probed with anti-myc, anti-PIF1 (Shen et al., 2008), or anti-RPT5 antibodies. For secondary antibody, anti-mouse (for myc) and anti-rabbit (for PIF1 and RPT5) antibodies (KPL Inc., Gaithersburg, MD) in 1:50 000 dilutions was used, and membranes were developed using a KPL Protein Detector LumiGLO Reserve Western Blotting Kit (#54–13–50) (KPL Inc., Gaithersburg, MA). The intensity of PIF1 and the control bands from each blot was quantified using ImageJ software and the PIF1 values were divided by the control values to make a ratio for each sample.

Chip Assay

The ChIP assay was performed on 3-day-old dark-grown seedlings expressing the TAP–PIF1 fusion protein as described (Moon et al., 2008). Antibody to the MYC tag was used to immunoprecipitate TAP–PIF1 and associated DNA fragments. DNA was amplified by real-time PCR using primers specific to the region containing the G-box element or control regions in *TROL*, *PIF4*, and *PBS27* genes (Supplemental Table 3).

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online*.

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