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The Basic Helix-Loop-Helix Transcription Factor PIF5 Acts on Ethylene Biosynthesis and Phytochrome Signaling by Distinct Mechanisms [W]

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PHYTOCHROME-INTERACTING FACTOR5 (PIF5), a basic helix-loop-helix transcription factor, interacts specifically with the photoactivated form of phytochrome B (phyB). Here, we report that dark-grown *Arabidopsis thaliana* seedlings overexpressing PIF5 (PIF5-OX) exhibit exaggerated apical hooks and short hypocotyls, reminiscent of the triple response induced by elevated ethylene levels, whereas *pif5* mutants fail to maintain tight hooks like those of wild-type seedlings. Silver ions, an ethylene receptor blocker, rescued the triple-response phenotype, and we show that PIF5-OX seedlings express enhanced levels of key ethylene biosynthesis enzymes and produce elevated ethylene levels. Exposure of PIF5-OX seedlings to prolonged continuous red light (Rc) promotes hypocotyl elongation relative to dark controls, the reciprocal of the Rc-imposed hypocotyl inhibition displayed by wild-type seedlings. In contrast with this PIF5-OX hyposensitivity to Rc, *pif5* mutant seedlings are hypersensitive relative to wild-type seedlings. We show that this contrast is due to reciprocal changes in phyB protein levels in prolonged Rc. Compared with wild-type seedlings, PIF5-OX seedlings have reduced, whereas *pif5* mutants have increased, phyB (and phyC) levels in Rc. The phyB degradation in the overexpressors depends on a functional phyB binding motif in PIF5 and involves the 26S proteasome pathway. Our data thus indicate that overexpressed PIF5 causes altered ethylene levels, which promote the triple response in darkness, whereas in the light, the interaction of photoactivated phyB with PIF5 causes degradation of the photoreceptor protein. The evidence suggests that endogenous PIF5 negatively regulates phyB-imposed hypocotyl inhibition in prolonged Rc by reducing photoreceptor abundance, and thereby photosensory capacity, rather than functioning as a signaling intermediate.

INTRODUCTION

Early seedling development is regulated by a complex interplay between the hormonal and light signaling pathways. Dark-grown (etiolated) seedlings have elongated hypocotyls and unexpanded cotyledons supported by an apical hook. The curvature of the hook provides protection for the cotyledons and the meristematic primordia and facilitates the movement of the seedling during soil penetration (Darwin and Darwin, 1881; Harpham et al., 1991). Premature hook opening and cotyledon expansion can be detrimental for the survival of a seedling submerged in compact soil. Previous studies have found that ethylene (C_2H_2), a gaseous hormone, plays a key role in regulating leaf expansion and shoot growth when tomato (*Solanum lycopersicum*) plants are subjected to differential soil compaction (Hussain et al., 1999). Ethylene, along with gibberillin and auxin, play important roles in hook formation by regulating differential cellular expansion in the apical region of the seedling (Li et al., 2004; Vriezen et al., 2004). Mutant seedlings lacking *CONSTITUTIVE TRIPLE RESPONSE1* (*CTR1*) function, a negative component of ethylene signaling, develop exaggerated hooks even when ethylene is absent (Guzman and Ecker, 1990; Kieber et al., 1993). On the other hand, *ETHYLENE-INSENSITIVE2* (*EIN2*) mutants fail to respond to ethylene and are incapable of forming exaggerated hooks even in the presence of ethylene (Roman et al., 1995). In a germinating seedling, ethylene is synthesized in the apical hook region (Goeschl et al., 1966; Taylor et al., 1988). The precursor of ethylene, 1-aminocyclopropane-1-carboxylic acid (ACC), is produced by the enzyme ACS (for ACC SYNTHASE) in a rate-limiting step (Zarembinski and Theologis, 1994; Bleecker and Kende, 2000; Wang et al., 2002). ACC is then oxidized by ACO (for ACC OXIDASE) to produce CO_2, HCN, and ethylene. ACS is a cytosolic enzyme with a short half-life whose activity is regulated at both the transcriptional and posttranscriptional levels (Liang et al., 1992; Zarembinski and Theologis, 1994; Bleecker and Kende, 2000; Wang et al., 2002). *Arabidopsis thaliana* has nine *ACS* genes; eight of these genes (*ACS2*, *ACS4* to *ACS9*, and *ACS11*) encode functional polypeptides, and one (*ACS1*) is nonfunctional (Arabidopsis Genome Initiative, 2000; Yamagami et al., 2003). ACS8 has significantly higher enzymatic activity

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than the other ACS proteins (Yamagami et al., 2003), and ethylene levels closely correlate with the expression of the *ACS8* gene, which is regulated by light, the circadian clock, and negative feedback from the ethylene pathway (Thain et al., 2004). While the circadian clock regulates the expression of multiple *ACS* genes, it has the most prominent effect on *ACS8* expression (Thain et al., 2004). In addition, Thain et al. (2004) showed that ethylene production is likely an output of the circadian clock and that changes in ethylene levels have no significant influence on clock function itself. *ACS* gene expression is also regulated by auxin, as treatment with indole-3-acetic acid induces the expression of all but *ACS7* and *ACS9* (Yamagami et al., 2003).

Light acts to rapidly repress ethylene activity and exerts influence in mediating differential growth responses with organ specificity (Li et al., 2004). Plants are sensitive to the quality, intensity, and duration of the light signal throughout their lives (Schäfer and Nagy, 2006). Deetiolation is characterized by the inhibition of hypocotyl cell elongation, along with hook opening, cotyledon cell expansion, and greening. The photosensory photoreceptors for red/far-red light (FRc), the phytochromes, function in the early dark-to-light transitions in growth and development (Quail, 2002a, 2002b). The five *Arabidopsis* phytochromes (phyA to phyE) are well characterized. Whereas phyA is the primary photoreceptor in continuous FRc, phyB plays a major role in continuous red light (Rc) (Quail et al., 1995; Whitelam and Devlin, 1997; Quail, 1998; Smith, 2000). Furthermore, different members of the phytochrome family act with partial organ specificity in red light. For example, the responsiveness of the apical organs (hook and cotyledons) is regulated by multiple phytochromes, including phyB, whereas the inhibition of hypocotyl cell elongation in red light is predominantly regulated by phyB (Tepperman et al., 2004).

PHYTOCHROME-INTERACTING FACTOR5 (PIF5) belongs to subfamily 15 of the basic helix-loop-helix (bHLH) class of transcription factors (Bailey et al., 2003; Khanna et al., 2004). Subfamily 15 consists of 15 members, including PIF1 (also called PIL5, for PIF3-LIKE5), PIF3, PIF4, PIF5 (PIL6), and PIF6 (Bailey et al., 2003; Toledo-Ortiz et al., 2003; Yamashino et al., 2003; Khanna et al., 2004). PIF1 has been implicated in chlorophyll biosynthesis (Huq et al., 2004). In addition, PIF1 is also thought to act as a repressor of some gibberellin biosynthetic genes as well as an activator of specific genes involved in gibberellin catabolism (Oh et al., 2006). It was shown that *pif1* mutant seedlings accumulate higher levels of gibberellin and germinate more readily (Oh et al., 2006). These data suggest that PIF1 may function as a node between gibberellin and light signaling. PIF1 interacts specifically with photoactivated (Pfr) forms of both phyA and phyB (Huq et al., 2004). The only other family member that binds both phyA (Pfr) and phyB (Pfr), and the first member of the PIF family to be identified, is PIF3 (Ni et al., 1998, 1999). Previous studies have suggested that PIF3 plays a role in chloroplast development (Monte et al., 2004). While *pif3* and *pif4* mutant seedlings are hypersensitive to red light, seedlings overexpressing *PIF3* or *PIF4* have elongated hypocotyls in red light and exhibit phenotypes similar to *phyB* mutants (Huq and Quail, 2002; Kim et al., 2003; Monte et al., 2004). PIF5 was identified as PIF3-LIKE6 (PIL6) based upon its sequence relatedness to PIF3 and was later named PIF5 as a bona fide phy-interacting factor (Yamashino et al., 2003; Khanna et al., 2004). In amino acid sequence comparisons of the At bHLH family, PIF5 is most closely related to PIF4. A previous study using a *pif5* mutant (*pil6-1*) and seedlings overexpressing *PIF5* showed that PIF5 functions negatively in phy-mediated pathways (Fujimori et al., 2004). PIF4 and PIF5 interact specifically with the photoactivated form of phyB through their active phytochrome binding (APB) motif (Khanna et al., 2004). In a previous study, we showed that a *PIF4* transgene carrying a functional APB motif is required to rescue the red light hypersensitivity phenotype of *pif4* mutant seedlings (Khanna et al., 2004). Furthermore, the magnitude of hyposensitivity to red light conferred by *PIF4* overexpression was correlated directly with the level of *PIF4* expression from the transgene carrying a functional APB motif (Khanna et al., 2004). These data suggested that increased levels of PIF4 reduced red light sensitivity similar to that of *phyB*-null seedlings and that this phenotype was somehow dependent upon the APB-mediated interaction of PIF4 with photoactivated phyB. Recent data have indicated that PIF4 and PIF5 protein levels decline rapidly in the light (Nozue et al., 2007; Shen et al., 2007). PIF4 and PIF5 were shown to function in promoting growth at the end of the night by integrating signals from light and the circadian clock (Nozue et al., 2007). The underlying mechanisms by which PIF4 and PIF5 promote growth are not known.

In this study, we have isolated plants carrying two new mutant alleles of *PIF5* and generated independent lines overexpressing *PIF5* to study PIF5 function during early deetiolation. We show that seedlings overexpressing PIF5 accumulate higher ethylene levels than wild-type seedlings and that overexpressed PIF5 possibly plays a role in regulating *ACS* transcript levels. We show that in etiolated seedlings, PIF5 function is required for the optimal maintenance of the apical hook and to inhibit the cellular expansion of cotyledons, which is important in protecting the meristematic primordia in seedlings emerging through the soil. We also show that the interaction of PIF5 and photoactivated phyB results in changes in phyB protein abundance in deetiolating seedlings, likely through the involvement of the 26S proteasome pathway. These results suggest that PIF5 can promote ethylene activity in the dark and increase growth in the light by decreasing phyB protein abundance.

RESULTS

PIF5 is a bHLH-type transcription factor of subfamily 15 and contains an APB motif near the N terminus, as shown in Figure 1A. In this study, we used three independent T-DNA insertional mutants: two new alleles, *pif5-1* and *pif5-2*, and a previously described allele, *pil6-1* (Fujimori et al., 2004) (designated *pif5-3*; Figure 1). While *pif5-1* retains some, albeit lower, levels of *PIF5* transcript, *pif5-2* and *pif5-3* appear to be null mutations (see Supplemental Figure 1 online).

Red Light Induces Hypocotyl Elongation in Seedlings Overexpressing PIF5

All three *pif5* mutant alleles are hypersensitive to red light, exhibiting shorter hypocotyls and larger cotyledons than wild-type

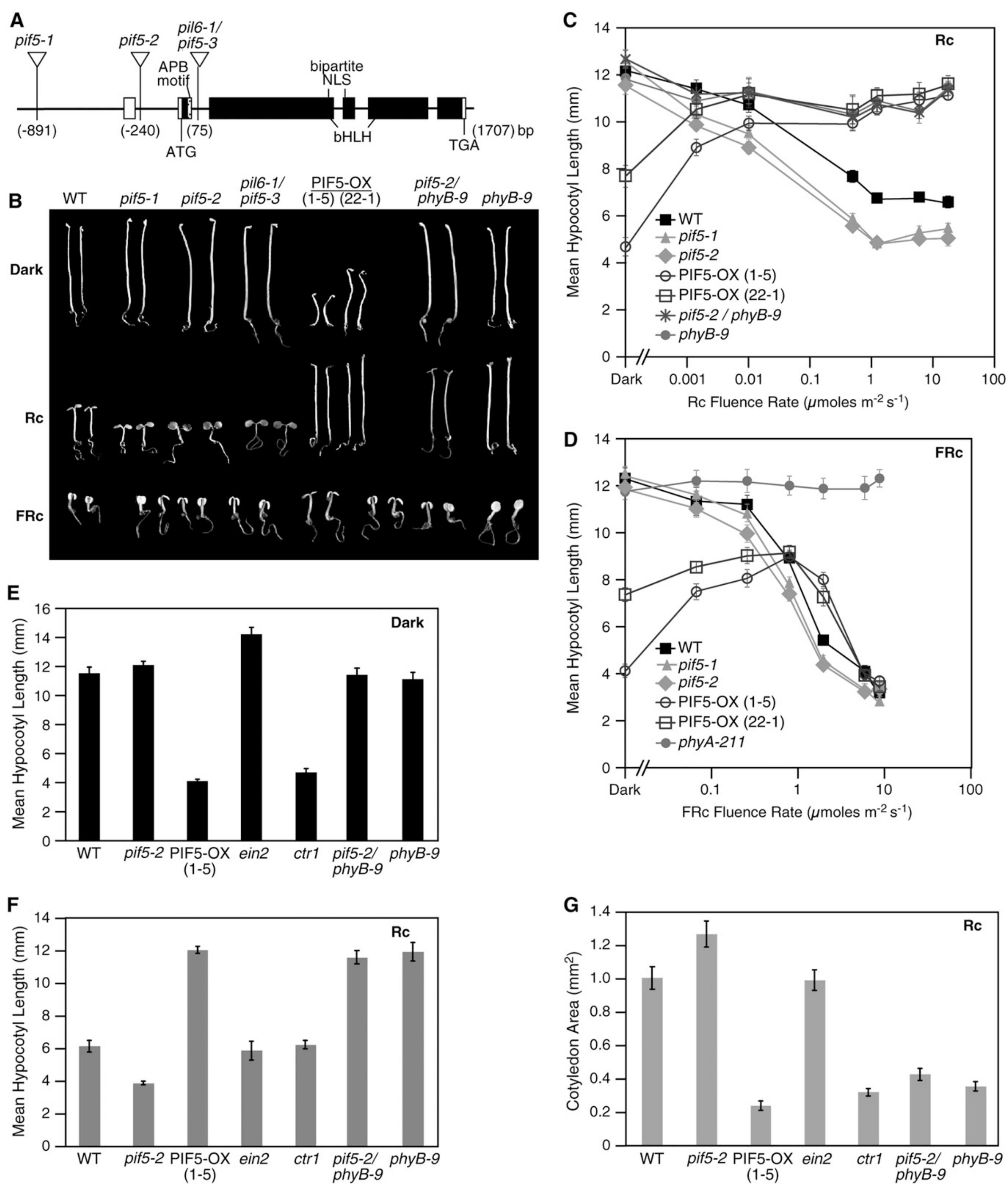

Figure 1. Red Light Induces Hypocotyl Elongation in Seedlings Overexpressing PIF5.

(A) *PIF5* gene structure. Positions of the insertional disruption sites in *pif5-1*, *pif5-2*, and *pif5-3* are indicated.

(B) Four-day-old seedlings of the three *pif5* mutants, two PIF5-OX lines, and a *pif5-2 phyB-9* double mutant are shown in comparison with Columbia (Col; wild type) and *phyB-9*. Seedlings were grown either in the dark for 4 d or in Rc or FRc for 3 d.

plants (Figure 1B). These data confirm the previous reports for *pil6-1* (*pif5-3*) (Fujimori et al., 2004). Conversely, transgenic lines overexpressing *PIF5* [two independent lines are shown, PIF5-OX (1-5) and PIF5-OX (22-1)] are strikingly hyposensitive to red light, displaying markedly longer hypocotyls and smaller cotyledons than the wild type (Figure 1B). Most surprisingly, however, the PIF5-OX lines grown in the dark have shorter, thicker hypocotyls as well as exaggerated apical hooks (Figure 1B). This phenotype is reminiscent of the triple response normally attributed to elevated ethylene levels, suggesting the possibility that etiolated PIF5-OX seedlings may have higher than normal ethylene levels or may be hypersensitive to the phytohormone.

Red light induces cellular expansion of hypocotyls in PIF5-OX seedlings, which is proportionate to the increase in red fluence rate: low fluence rate Rc leads to increased hypocotyl length with increasing fluence rate up to 1 $\mu mol \cdot m^{-2} \cdot s^{-1}$, reaching the hypocotyl length of dark-control wild-type seedlings (Figure 1C). This hypocotyl phenotype of PIF5-OX seedlings grown in darkness and red light is unprecedented and is clearly opposite to that of the wild-type seedlings (Figure 1C). The PIF5-OX seedlings showed an increase in hypocotyl length with increasing FRc fluence rate up to 1 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Further increases in fluence rate resulted in the inhibition of hypocotyl elongation similar to the growth inhibition in wild-type seedlings (Figure 1D). On the other hand, the *pif5* mutant seedlings are slightly hypersensitive to FRc (Figure 1D). The *pif5* mutant seedlings exhibit relatively greater hypersensitivity to red light than to FRc (Figures 1C and 1D).

Altering *PIF5* Transcript Levels Causes Phenotypes Associated with Ethylene and Light Signaling

The phenotype observed for dark-grown PIF5-OX seedlings suggests possible ethylene involvement. To further analyze this possibility, hypocotyl lengths of the etiolated *pif5* mutant and PIF5-OX seedlings were compared with those of *ein2* and *ctr1* mutants. Whereas *ein2* mutant seedlings are insensitive to ethylene and are slightly taller than wild-type seedlings in the dark, the *ctr1* mutants are constitutively responsive to ethylene and are shorter in the dark, similar to the PIF5-OX seedlings (Figure 1E). These data suggest that PIF5-OX seedlings may be more sensitive to ethylene and/or may have higher ethylene levels. The *pif5* mutant seedlings do not show a distinctive hypocotyl phenotype in the dark. There are two possible explanations for the lack of observable hypocotyl phenotype in dark-grown *pif5* mutant seedlings: either endogenous PIF5 has no intrinsic role in ethylene physiology and the triple response phenotype observed for PIF5-OX seedlings is a result of ectopic overexpression of PIF5, or the *pif5* mutant retains normal growth in the dark because of functional overlap between endogenous PIF5 and one or more other proteins.

Changes in ethylene levels or sensitivity cannot completely explain the range of phenotypes observed for PIF5-OX and *pif5* mutants in red light, as there is little or no effect of *ein2* and *ctr1* mutations on hypocotyl growth under these conditions (Figure 1F). Expansion of cotyledons, however, is strongly affected in *ctr1* mutant seedlings grown in red light (Figure 1G). Either the *pif5* mutation or PIF5 overexpression causes reciprocal effects on hypocotyl growth and cotyledon area in seedlings grown in red light, which is characteristic of phenotypes attributed to lesions in the photomorphogenic pathway (Khanna et al., 2006). The *ein2* and *ctr1* mutant seedlings do not exhibit phenotypes typical of seedlings defective in light signaling, consistent with the specific function of these genes in ethylene signaling. These data further suggest that the development of the apical region and the hook (hook opening), as well as cotyledon separation and expansion in red light, are more sensitive to changes in ethylene activity and that inhibition of hypocotyl elongation in red light is relatively less responsive to ethylene. The hypocotyl length of the *pif5-2 phyB-9* double mutant resembles that of the *phyB* single mutant in red light (Figure 1), indicating that the hypocotyl phenotype of the *pif5* mutant is dependent upon functional phyB. Collectively, these data suggest that overexpression of PIF5 affects two distinct pathways: one specific to dark-grown seedlings and possibly due to increased ethylene sensitivity or ethylene levels, and a second that negatively affects phyB-mediated photomorphogenesis.

Effects of the *pif5* Mutation and *PIF5* Overexpression on Ethylene-Related Responses

Ethylene is known to affect hook opening and cotyledon separation. We compared the degree of apical hook opening and cotyledon separation in *pif5* mutant, PIF5-OX, and wild-type seedlings after growth in the dark or red light. We found that *pif5* mutant seedlings exhibit a hypersensitive response, whereas PIF5-OX seedlings are hyposensitive to red light in hook opening and cotyledon separation compared with wild-type seedlings (Figure 2A). Interestingly, 4-d-old *pif5* mutant seedlings have larger hook angles in the dark compared with wild-type seedlings (Figure 2A). On the other hand, hook angles are similar in the wild type and *pif5* mutants in 2-d-old seedlings (Figure 2A). This shows that the *pif5* mutants are defective in the normal asymmetric cellular elongation in the hook in the dark, suggesting that the *pif5* mutants may be less responsive to the normal ethylene levels regulating hook opening, or that they may have lower ethylene levels. These results suggest the possibility that the ethylene pathway regulating hook opening and cotyledon separation may be overactive in PIF5-OX seedlings and possibly less active in the *pif5* mutant. Next, we treated the seedlings either with 100 μM $AgNO_3$ (which competes with ethylene and blocks

Figure 1. (continued).

(C) and **(D)** Fluence rate response curves of seedlings grown in Rc **(C)** or FRc **(D)**.

(E) and **(F)** Mean hypocotyl lengths of 4-d-old seedlings grown in the dark **(E)** or Rc **(F)** compared with those of *ein2* and *ctr1* mutants.

(G) Cotyledon areas of seedlings grown in Rc.

Fluence rates of Rc (7 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and FRc (2 $\mu mol \cdot m^{-2} \cdot s^{-1}$) were used (where not indicated). Approximately 30 seedlings of each genotype were used for each analysis. SE values were determined and plotted as shown.

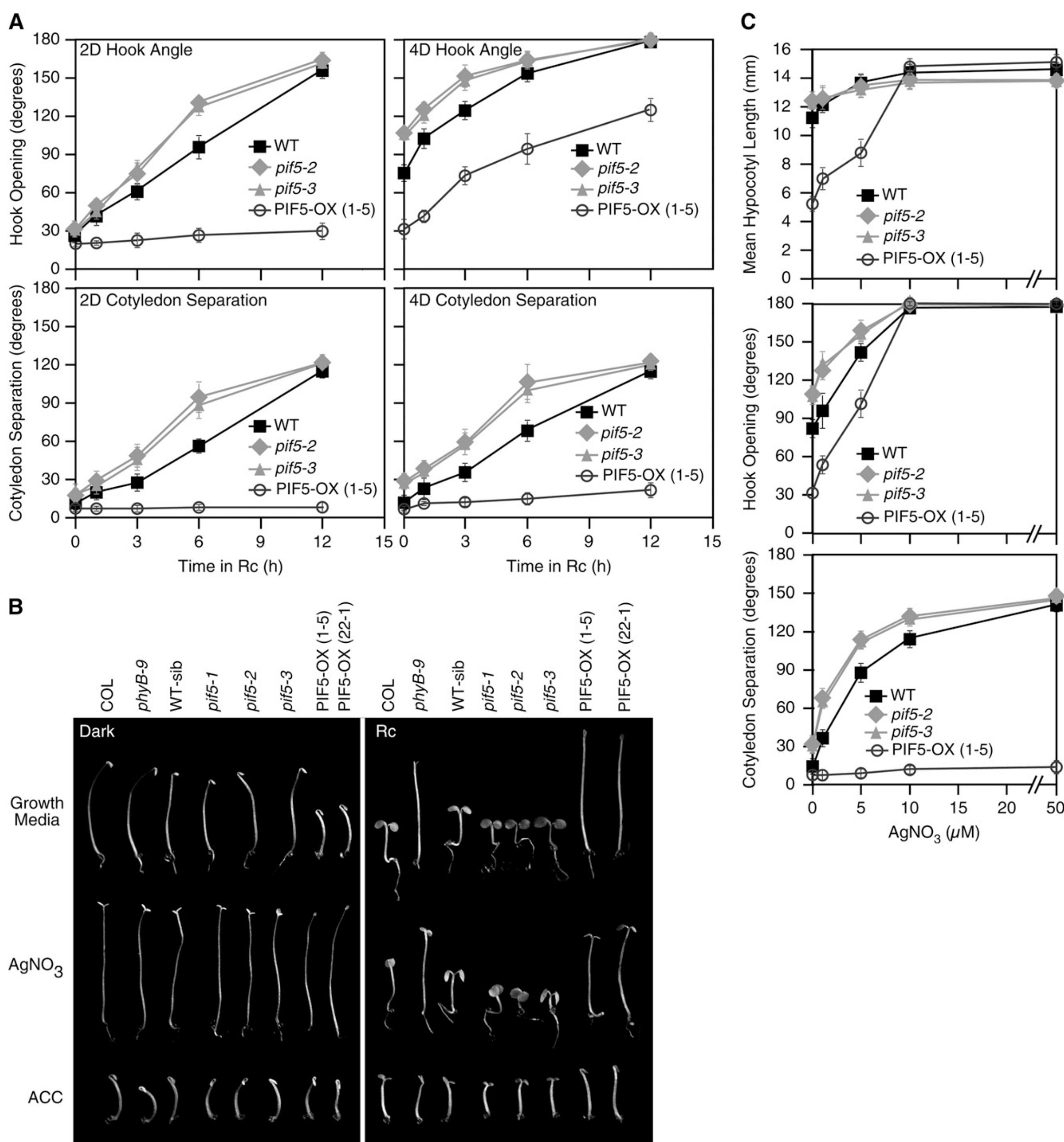

Figure 2. PIF5-OX and *pif5* Mutant Seedlings Exhibit Abnormal Hook Opening and Cotyledon Separation.

(A) Hook opening (top panels) and cotyledon separation (bottom panels) were determined for 2-d-old (left panels) and 4-d-old (right panels) etiolated seedlings transferred to Rc for up to 12 h.

(B) Four-day-old seedlings either grown in the dark (left panel) or treated with Rc (right panel) for 3 d and grown on growth medium with or without AgNO3 (50 μM) or ACC (20 μM).

(C) Etiolated seedlings were grown on growth medium supplemented with different concentrations of AgNO3. Hypocotyl length (top panel), hook opening (middle panel), and cotyledon separation (bottom panel) were determined.

Approximately 30 seedlings of each genotype were used for each analysis. SE values were determined and plotted as shown.

accessibility to ethylene receptors), a potent inhibitor of the ethylene pathway, or with 20 μM ACC (a precursor for ethylene synthesis) (Figure 2B). As expected, treatment with $AgNO_3$ resulted in hook opening and cotyledon expansion in the dark (Figure 2B, middle left panel). Interestingly, treatment with $AgNO_3$ is sufficient to rescue the triple response phenotype observed for PIF5-OX seedlings (Figure 2B, middle left panel). By contrast, $AgNO_3$ treatment did not rescue the hypocotyl phenotypes observed in red light (Figure 2B, middle right panel). Treatment with $AgNO_3$ increased cotyledon size in seedlings that exhibited partially etiolated phenotypes in response to red light (Figure 2B, middle right panel). Treatment with ACC caused exaggerated hooks in the dark; seedlings treated with ACC showed a typical triple response, characteristic of high ethylene activity (Figure 2B, bottom left panel). In addition, ACC-treated seedlings grown in red light had separated cotyledons and exhibited inhibition of hypocotyl elongation (Figure 2B, bottom right panel). Furthermore, *phyB* mutant seedlings treated with ACC and grown in red light are short, like wild-type seedlings, suggesting that the ACC-induced inhibition of hypocotyl elongation is either through a mechanism distinct from the phyB signaling pathway or that ethylene acts downstream of phyB in regulating hypocotyl growth (Figure 2B). The effect of ACC treatment on *phyB* mutant seedlings may not be unexpected, because *phyB* mutants grown in red light behave like etiolated seedlings.

The phenotype rescue of etiolated PIF5-OX seedlings by treatment with $AgNO_3$ suggests that PIF5-OX seedlings have increased ethylene activity at the ethylene receptor, which can be blocked by treatment with silver ions. To study whether the *pif5* mutant and PIF5-OX seedlings have altered sensitivities to ethylene, we grew etiolated seedlings on different concentrations of $AgNO_3$ (0 to 50 μM) or ACC (0 to 20 μM). All of the seedlings showed some response to $AgNO_3$ treatment (Figure 2C) and ACC treatment (data not shown). With respect to hypocotyl length, *pif5* mutant and wild-type seedlings responded similarly. In addition, the hypocotyl lengths of the PIF5-OX seedlings were completely rescued by treatment with 10 μM $AgNO_3$ (Figure 2C, top panel). With regard to hook opening, seedlings responded similarly to $AgNO_3$ treatment, maintaining their initial differences until 10 μM $AgNO_3$, at which point these seedlings had completely open hooks (Figure 2C). Finally, *pif5* mutant and wild-type seedlings showed similar responses to $AgNO_3$ in the degree of cotyledon separation, with *pif5* mutant seedlings retaining greater separation at all $AgNO_3$ concentrations except 50 μM $AgNO_3$, at which point *pif5* mutant and wild-type seedlings had the same degree of cotyledon separation. By contrast, PIF5-OX seedlings showed only a very weak response to $AgNO_3$ with regard to cotyledon separation. Even at 50 μM $AgNO_3$, PIF5-OX cotyledons remained mostly appressed (Figure 2C). Cotyledons on most of the PIF5-OX seedlings remained unexpanded (as can be seen in Figure 2B, middle left panel). However, the hypocotyls and hooks of these PIF5-OX seedlings responded to treatment with silver (Figure 2C). These data suggest the possibility that the *pif5* mutant and PIF5-OX seedlings are responsive to $AgNO_3$ treatment but may have altered ethylene levels compared with the wild type. The *pif5* mutant may have reduced ethylene levels, whereas the PIF5-OX seedlings are predicted to have higher levels of ethylene.

Effects of the *pif5* Mutation and *PIF5* Overexpression on *ACS* Transcript and Ethylene Levels

ACS is the rate-limiting enzyme involved in ethylene biosynthesis. There are eight functional *ACS* genes in *Arabidopsis* (Yamagami et al., 2003). ACS4, ACS5, ACS8, and ACS9 are more closely related to each other in amino acid sequence comparisons than to any other ACS proteins. Expression of *ACS8* is controlled by the circadian clock and is likely responsible for the rhythmic changes in ethylene levels (Thain et al., 2004). *ACS8* expression is also regulated by negative feedback from the ethylene pathway. To analyze whether the *pif5* mutant and overexpressing seedlings had altered *ACS* transcript levels that were not due to feedback within the ethylene pathway, we examined the transcript levels of several *ACS* genes in *pif5* mutant and PIF5-OX seedlings as well as in wild-type seedlings treated with 50 μM $AgNO_3$ or 20 μM ACC (Figure 3; see Supplemental Figure 2 online). Etiolated seedlings overexpressing PIF5 had remarkably higher levels of *ACS4* and *ACS8* transcripts compared with those found in the wild type (Figures 3A and 3C). PIF5-OX had *ACS4* transcript levels significantly higher than in wild-type seedlings treated with ACC (Figure 3A), indicating that this increase in *ACS4* transcript is likely not due to increased ethylene levels alone. These data suggest a possible role for PIF5 in the regulation of *ACS4* transcript; however, *pif5* mutant and wild-type seedlings treated with $AgNO_3$ also had slightly elevated levels of *ACS4*, making the results inconclusive for this gene.

The transcript levels of *ACS8* responded negatively, as expected, to treatment of wild-type seedlings with ACC, exhibiting the documented feedback from ethylene signaling (Thain et al., 2004). PIF5-OX seedlings had elevated levels of *ACS8* transcript, whereas *pif5* mutant seedlings had slightly reduced levels of *ACS8* message compared with the wild-type seedlings (Figure 3C). These data suggest that *ACS8* transcript levels respond negatively to elevated ethylene levels but that the *ACS8* levels are correlated with increased *PIF5* transcript levels in the PIF5-OX seedlings. The relatively small changes observed in *ACS* transcript levels in *pif5* mutant compared with wild-type seedlings make it difficult to conclude that endogenous PIF5 plays a role in regulating *ACS* transcript levels. The lack of significant differences in *ACS* levels in the *pif5* mutant may possibly be attributed to functional redundancy with one or more other factors, or it may suggest that endogenous PIF5 has no role in regulating these genes and that the differences observed in PIF5-OX seedlings were caused by the ectopic overexpression of PIF5.

We examined these seedlings for ethylene levels and found that etiolated PIF5-OX seedlings have approximately fourfold higher ethylene levels than etiolated wild-type seedlings (Figure 3E). However, in Rc-grown seedlings, ethylene levels in PIF5-OX seedlings did not differ in a statistically significant manner from those of wild-type seedlings (Figure 3F). By contrast, the *pif5* mutant seedlings contained ethylene levels similar to those of wild-type seedlings under dark and red light (Figures 3E and 3F).

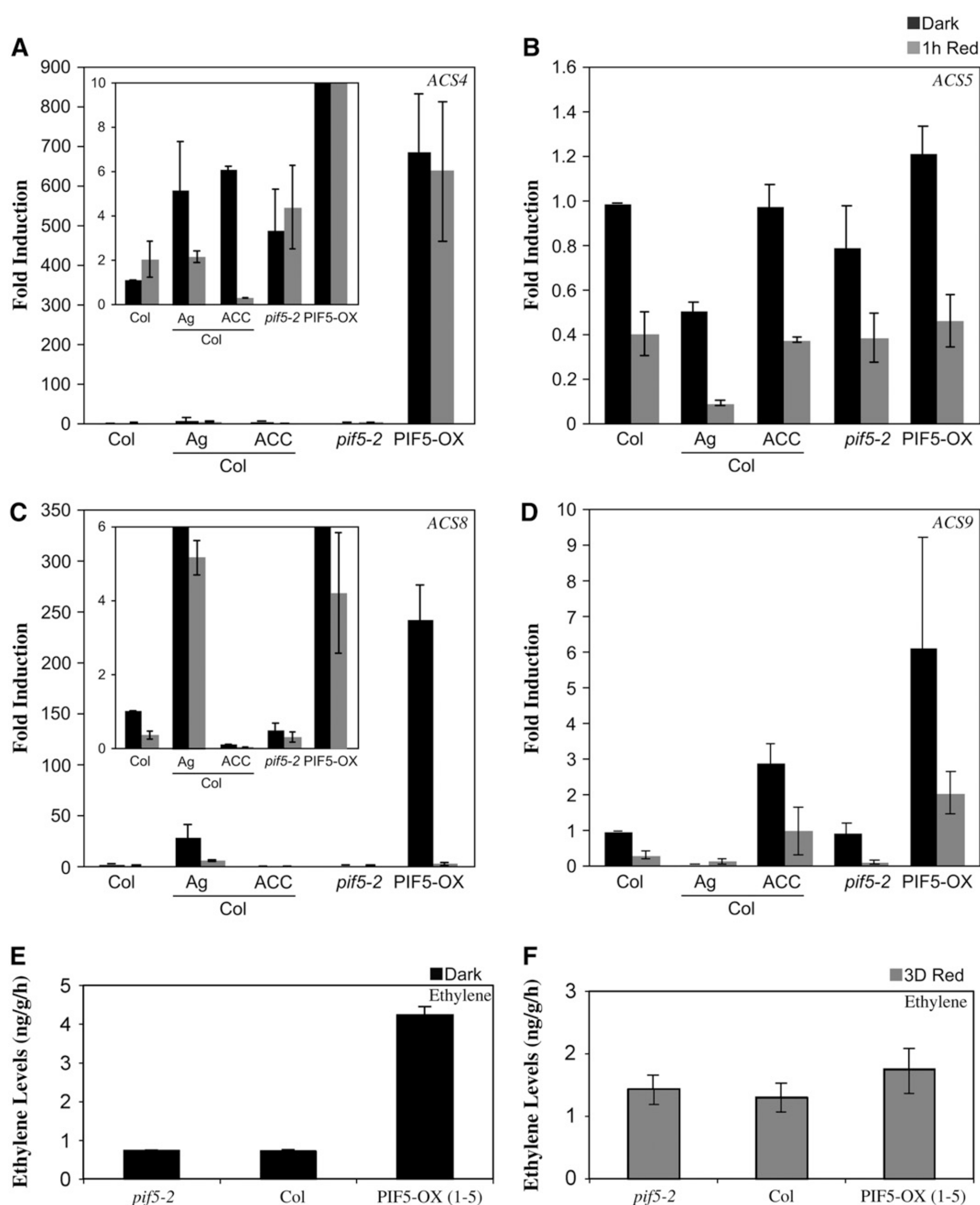

Figure 3. Overexpressed PIF5 Regulates *ACS8* and Possibly Other *ACS* Transcript Levels as Well as Ethylene Levels in Dark-Grown Seedlings.

Quantitative RT-PCR using RNA from 4-d-old seedlings grown in the dark or exposed to Rc for 1 h. Data are shown for *ACS4* **(A)**, *ACS5* **(B)**, *ACS8* **(C)**, and *ACS9* **(D)**. These *ACS* genes are most closely related to each other in amino acid sequence comparisons of all of the ACS proteins. Col (wild-type) seedlings grown on AgNO3 (50 μM) or ACC (20 μM) were used to examine the feedback effects of ethylene signaling on the transcript levels of these *ACS* genes. Insets show different scales to visualize small changes. Cycle threshold values were used to calculate fold induction with Col dark values set to 1. Values from three biological replicates are plotted with SE. Ethylene levels produced in *pif5-2* and PIF5-OX (1-5) were measured and compared with the ethylene levels produced in Col using etiolated seedlings **(E)** or seedlings grown in red light for 3 d **(F)**. Average values from three biological replicates are plotted with SE.

These data provide an explanation for the triple response phenotype of PIF5-OX seedlings as well as the aberrant hook opening and cotyledon separation observed in PIF5-OX seedlings during early seedling development (Figures 1 and 2). However, in the *pif5* mutants, while the relatively small changes in *ACS* transcript levels (Figure 3C) correlate with the subtle phenotypes observed in hook opening and cotyledon separation (Figure 2), the lack of any detectable difference in ethylene levels does not support the conclusion that endogenous PIF5 plays a role in regulating ethylene levels (see Discussion).

Effects of the *pif5* Mutation and *PIF5* Overexpression on phy Protein Levels in Red Light

We examined phytochrome protein levels in PIF5-OX and *pif5* mutant seedlings during early deetiolation and compared the levels with those found in wild-type seedlings (Figure 4; see Supplemental Figure 3 online). While phyA protein levels declined in all seedlings in response to red light (Figures 4A and 4E), the PIF5-OX seedlings had higher phyA protein levels in the dark (Figure 4A), and the protein persisted for ~3 h longer in PIF5-OX seedlings (Figure 4E). On the other hand, phyB protein levels in the dark were relatively similar to those in wild-type seedlings (Figure 4B). Surprisingly, phyB protein levels were significantly decreased in PIF5-OX seedlings relative to wild-type seedlings

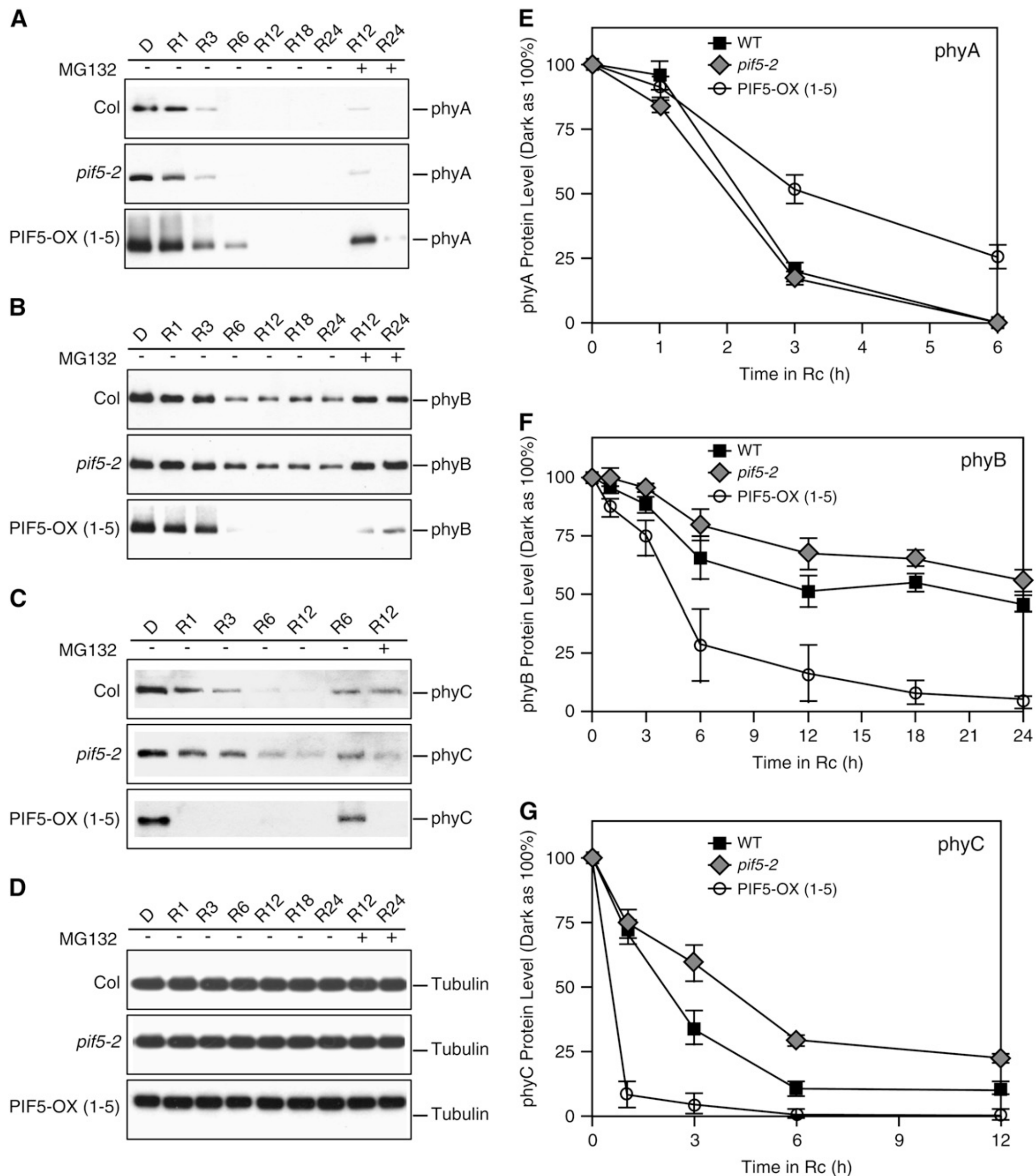

Figure 4. Phytochrome Protein Levels in PIF5-OX and *pif5* Mutants.

Immunoblot analysis of phy protein levels. Four-day-old seedlings were treated with Rc for the specified times. Some of the seedlings were treated with MG132 (to block the 26S proteasome pathway). Quantifications of immunoblots are shown at right: phyA (**[A]** and **[E]**), phyB (**[B]** and **[F]**), phyC (**[C]** and **[G]**), and tubulin **(D)**. Values from three biological replicates are plotted with SE.

after 6 h in Rc, whereas *pif5* mutants had higher phyB protein levels than wild-type seedlings (Figures 4B and 4F). A similar effect was observed for phyC levels. It has been shown that *phyB* mutant seedlings have reduced phyC protein levels and that phyB stabilizes phyC in the light (Hirschfeld et al., 1998; Sharrock and Clack, 2002). Hence, it is not surprising that phyC protein levels are also reduced rapidly in PIF5-OX seedlings grown in Rc and that *pif5* mutants accumulate higher levels of phyC along with higher phyB levels (Figures 4C and 4G). PIF5-OX seedlings may also contain slightly lower than wild-type phyD levels, but phyE levels were similar to those found in wild-type seedlings (see Supplemental Figure 3 online). Furthermore, treatment with MG132 (an inhibitor of the 26S proteasome pathway) blocks phy protein degradation (Figures 4A to 4C; see Supplemental Figure 4 online). This result indicates that the 26S proteasome pathway is involved in phytochrome degradation in these seedlings. These changes in phytochrome protein levels in *pif5* mutant and PIF5-OX seedlings are most likely not due to changes in *PHY* transcript levels in the light (see Supplemental Figure 5 online). There was no significant difference in *PHYA* transcript levels in these seedlings (see Supplemental Figure 5A online). *PHYB* and *PHYD* transcript levels in dark-grown PIF5-OX seedlings were slightly higher (see Supplemental Figures 5B and 5D online), but there was no difference in phyB protein levels in dark-grown seedlings (Figure 5A), and these seedlings contained relatively lower than wild-type phyD protein levels in the dark (see Supplemental Figure 3A online). These data suggest that the observed changes in phy protein levels are likely due to altered phy protein stability in red light and not to changes in transcript levels.

Endogenous PIF5 Negatively Regulates Rc-Imposed Hypocotyl Inhibition Indirectly by Reducing phyB Photoreceptor Levels in Prolonged Irradiations

Since the hypocotyl length measurements were performed with 4-d-old etiolated seedlings or seedlings exposed to prolonged Rc for 3 d (Figure 1), we examined these seedlings in a quantitatively rigorous manner for phyA and phyB protein levels. We performed quantitative protein gel blot analysis by determining the linear range for intensity values to calculate relative protein levels, as described in Methods. Seedlings that were grown in Rc for 3 d retained 1.6-fold higher levels of phyB in the *pif5* mutant background and, conversely, contained significantly (5-fold) lower amounts of phyB in the PIF5-OX background (Figure 5B). It has been shown that the amount of phyB is an important and sensitive determinant of the magnitude of light inhibition of hypocotyl length (Wester et al., 1994). Here, we show that under prolonged Rc, the PIF5-OX seedlings have significantly lower phyB levels and that these seedlings are hyposensitive to this irradiation treatment, whereas, conversely, the *pif5* mutant seedlings contain elevated phyB levels and exhibit hypersensitivity to the prolonged red light signal (Figure 5C). Together, these results indicate that endogenous PIF5 negatively regulates Rc-imposed hypocotyl inhibition by reducing phyB protein levels under these conditions. The altered phyB photoreceptor levels in the *pif5* mutant and the PIF5-OX seedlings thus appear responsible for causing the observed hypersensitivity and hyposensitivity, respectively, to the Rc signal in these seedlings through otherwise normal phyB activity. Hence, the potential for PIF5 to regulate seedling responsiveness to prolonged Rc appears to be an indirect one, by feedback on the levels of the phyB photoreceptor itself, rather than PIF5 participating downstream directly in the phyB transduction pathway regulating hypocotyl growth.

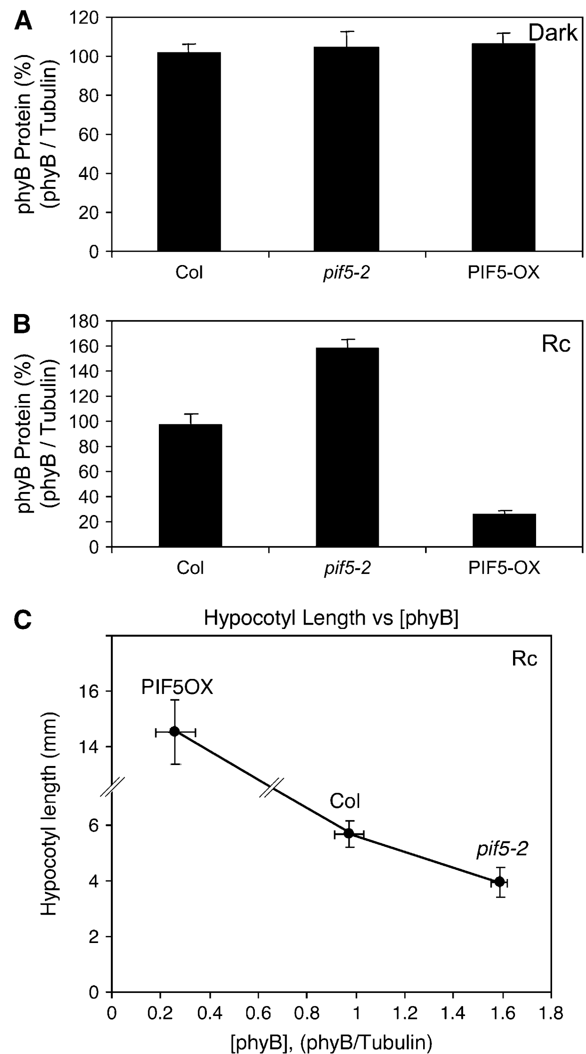

Figure 5. Endogenous PIF5 Negatively Regulates Rc-Imposed Hypocotyl Inhibition Indirectly by Reducing phyB Photoreceptor Levels in Prolonged Irradiations.

(A) Relative phyB protein levels (phyB/tubulin) in 4-d-old Col, *pif5-2* mutant, and PIF5-OX seedlings grown in darkness. Immunoblots of phyB and tubulin from three biological replicates were quantified using dilution series with levels in Col set to 100% (see Supplemental Figure 7B online).
(B) Relative phyB protein levels in 4-d-old Col, *pif5-2* mutant, and PIF5-OX seedlings grown in Rc for 3 d. Quantification was done as described for **(A)**. Seedlings grown in Rc for 3 d contained relatively lower phyB protein levels compared with etiolated seedlings (see Supplemental Figure 7A online).
(C) Mean hypocotyl lengths of 4-d-old Col, *pif5-2* mutant, and PIF5-OX seedlings grown in Rc for 3 d were measured and plotted against relative phyB protein levels in these seedlings (obtained in **[B]**).

These data further support our previous conclusion that the seedling phenotypes observed in red light for PIF5-OX and *pif5* mutant seedlings are caused by changes in phyB protein levels, whereas altered ethylene levels are responsible for the phenotypes observed in etiolated seedlings.

The APB Motif Is Involved in the Depletion of phyB Protein by Light in Seedlings Overexpressing PIF5

Previously, we reported that *pif4* mutant seedlings, which are hypersensitive to red light, could be complemented by a *PIF4* transgene carrying a functional APB motif but not by a *PIF4* (mAPB) transgene (carrying a G35A mutation within the APB motif, incapable of interaction with phyB) (Khanna et al., 2004). Like PIF4, the red light phenotypes exhibited by the PIF5-OX seedlings are dependent upon APB activity. PIF5-OX (mAPB) seedlings (carrying an equivalent G37A mutation) (Khanna et al., 2004) exhibit the ethylene-related triple response in the dark but lack the elongated hypocotyl phenotype in red light (Figure 6A). This provides additional evidence that the PIF5-OX effect in Rc is exerted independently of the ethylene system. Since the PIF5-OX (mAPB) seedlings respond normally to red light, we analyzed PIF5-OX (mAPB) seedlings for phyB protein levels. Interestingly, as in wild-type seedlings, phyB protein was detectable in PIF5-OX (mAPB) seedlings after 6 h in Rc (Figure 6B). Further analysis revealed that the PIF5-OX (mAPB) seedlings grown in red light for 6 h or longer contain higher phyB protein levels than PIF5-OX seedlings carrying a functional APB motif (Figure 6C). Treatment of PIF5-OX (mAPB) seedlings with MG132 blocked phyB protein degradation similarly to that in the wild-type seedlings (Figure 6B). These data indicate that the 26S proteasome–mediated depletion of phyB protein in PIF5-OX seedlings is dependent upon APB activity. The residual phyB degradation observed in PIF5-OX (mAPB) seedlings is comparable to that in wild-type seedlings. This is entirely expected: because the PIF5 overexpressors are in the wild-type background, degradation of phyB in these seedlings may be due to endogenous PIF5 and/or other PIFs. Interestingly, like the PIF5-OX seedlings, 4-d-old dark-grown PIF5-OX (mAPB) seedlings contain higher than wild-type levels of phyA protein (see Supplemental Figure 6A online). By contrast, like the wild-type seedlings, the PIF5-OX (mAPB) seedlings exposed to Rc for 3 d contain higher phyB protein levels than the PIF5-OX seedlings (see Supplemental Figure 6B online). It is possible that the higher phyA protein levels found in etiolated seedlings overexpressing PIF5 with or without functional APB are a consequence of increased ethylene activity in these seedlings.

DISCUSSION

At least one-third of subfamily 15 of the *Arabidopsis* bHLH class of transcription factors consists of proteins that have been identified as PHYTOCHROME-INTERACTING FACTORS: PIF1 and PIF3 to PIF6 (Bailey et al., 2003; Yamashino et al., 2003; Khanna et al., 2004). All of the PIF proteins are characterized by their ability to specifically interact with the Pfr form of phytochrome. They all interact with phyB (Pfr) via the APB motif that is necessary and sufficient for their ability to specifically bind phyB

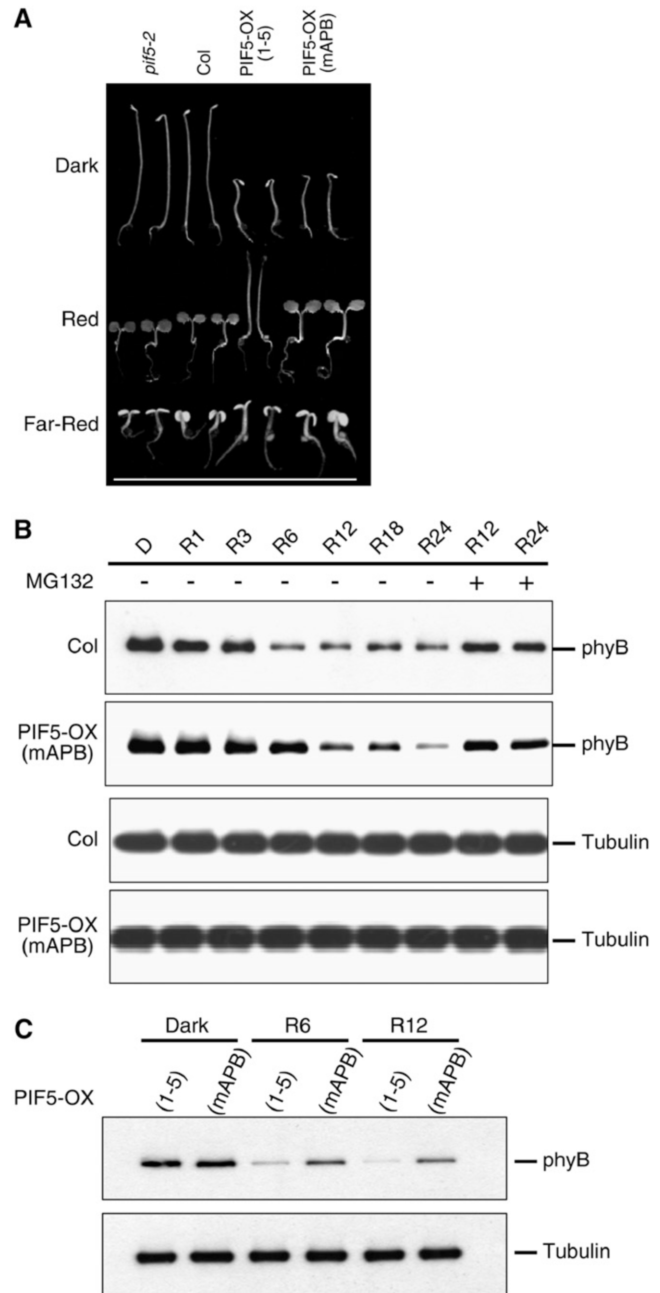

Figure 6. PIF5-OX (mAPB) Seedlings Carrying a Single G37A Mutation in the APB Motif Exhibit Normal Hypocotyl Response to Red Light and Contain Detectably Higher phyB Protein Levels Than the PIF5-OX Seedlings after 3 d in Rc.

(A) Four-day-old *pif5-2* mutant, Col, PIF5-OX (1-5), and PIF5-OX (mAPB) seedlings grown in the dark or exposed to Rc or FRc for 3 d.
(B) Immunoblot analysis of phyB protein levels in 4-d-old Col and PIF5-OX (mAPB) seedlings treated with red light and MG132 as indicated.
(C) Comparison of phyB protein levels between seedlings overexpressing PIF5 with either a functional APB motif [PIF5-OX (1-5)] or the mutated APB motif [PIF5-OX (mAPB)]. Representative immunoblots from three biological replicates are shown. Tubulin was used as a loading control.

(Pfr) (Khanna et al., 2004). PIF1 and PIF3 are the only bHLH class transcription factors that have been shown to interact with phyA (Pfr) (Ni et al., 1998; Huq et al., 2004; Al-Sady et al., 2006). Whereas interaction between PIF1 and phyA is detectable in pull-down assays, PIF3 interaction with phyA is ~10-fold weaker than its interaction with phyB (Ni et al., 1998, 1999; Huq et al., 2004). A recent study showed that phyA interacts with PIF3 at the active phytochrome A binding site found downstream of the APB motif in PIF3 (Al-Sady et al., 2006). This domain, however, is not conserved in any of the other PIF proteins, and it remains to be seen whether PIF1 has a similar or a unique domain responsible for its interaction with phyA (Pfr). Both PIF4 and PIF5 have been shown to specifically bind phyB (Pfr) through their APB motifs (Khanna et al., 2004), and they fail to bind any of the other phytochromes in in vitro immunoprecipitation assays (Huq and Quail, 2002; Khanna et al., 2004; Shen et al., 2007). Unlike *PIF3*, *PIF5* transcript levels are regulated by the circadian clock (Fujimori et al., 2004) and are induced by more than twofold after 1 h of growth in red light (see Supplemental Figure 1 online). However, PIF5 protein is rapidly degraded within 1 h in Rc (Shen et al., 2007). This response is similar to the light-induced degradation reported for PIF3 (Bauer et al., 2004; Park et al., 2004; Al-Sady et al., 2006). The data presented here raise the possibility that the APB-mediated interaction between PIF5 and phyB (Pfr) may result in the codegradation of phyB. However, PIF5 appears to degrade rapidly within 1 h, whereas phyB levels are detectable for up to 6 h in the light. Possible explanations for this discrepancy might include independent degradation pathways or differences in the ability to detect these proteins dependent upon the sensitivities of their respective antibodies.

PIF5 has been implicated as a negative regulator of red light–mediated photomorphogenesis (Fujimori et al., 2004). Our data support this conclusion and further demonstrate that PIF5 plays an important role in the developmental transition of the apical region (apical hook and cotyledons) during early seedling establishment. It has been shown that ethylene and light act antagonistically to regulate *HOOKLESS1*, which controls Auxin Response Factor2 protein levels to effect differential cell growth in the apical region of the hypocotyl (Li et al., 2004). Transcript levels of *ACS* genes, encoding enzymes involved in ethylene biosynthesis, are key determinants of ethylene production (Peck et al., 1998; Wang et al., 2002). We show that overexpression of PIF5 causes elevated expression of *ACS4*, *ACS8*, and possibly other *ACS* genes (Figure 3). The PIF5-OX seedlings exhibit the characteristic triple response in the dark and have higher ethylene levels (Figure 3E).

During the course of this study, Foo et al. (2006) reported that in the garden pea (*Pisum sativum*), light-grown *phyA phyB* mutants produce more ethylene than wild-type plants and that treatment of these mutants with aminoethoxy vinylglycine, an ethylene biosynthesis inhibitor, rescued many of the *phyA phyB* mutant phenotypes. They suggested a role for phytochromes in pea to regulate ethylene levels in the light to prevent inhibitory effects of ethylene on vegetative growth (Foo et al., 2006). They further suggested that some phytochrome-dependent responses are mediated by changes in ethylene levels (Foo et al., 2006). The mechanism of the phytochrome regulation of ethylene levels, however, is not known. We show that overexpression of PIF5 in *Arabidopsis* can increase ethylene production in seedlings growing in darkness. We did not observe any difference in ethylene levels between the *pif5* mutant and wild-type seedlings (Figures 3E and 3F). Thus, our data do not establish a role for endogenous PIF5 in regulating ethylene levels. However, the capacity for overexpressed PIF5 to affect the etiolated seedling phenotype through enhanced ethylene synthesis is not exhibited by any of the other closely related phy-interacting bHLH proteins that have been overexpressed, including PIF1 (Shen et al., 2005), PIF3 (Al-Sady et al., 2006), and PIF4 (Huq and Quail, 2002). This indicates that this capacity appears to be specific to PIF5, consistent with the possibility that this reflects an intrinsic function of this protein. It remains possible, therefore, that endogenous PIF5 may function in regulating ethylene levels but that this is masked in the *pif5* mutant, either because of functional redundancy with other factors or because subtle differences in ethylene levels localized in the apical organs are difficult to detect with whole seedlings. Consistent with this possibility, recent studies with insertional mutants of multiple members of the nine-member *ACS* gene family in *Arabidopsis* show that simultaneous mutation of several members is necessary before a decrease in total seedling ethylene levels is detectable (A. Tsuchisaka and A. Theologis, unpublished data). The high degree of redundancy between these genes indicated by this observation increases the probability of similar redundancy among potential regulators of these genes, such as PIF5. In addition, all members of the family exhibit both unique and overlapping spatiotemporal expression patterns during development, suggesting the possibility of localized regulation of ethylene levels in specific tissues or cell types not detectable by whole seedling ethylene measurements (Tsuchisaka and Theologis, 2004). Similarly, differential growth in the apical hook is marked by a spatially defined pattern of gene expression, including that of the ethylene biosynthetic enzyme At *ACO2*, which accumulates in cells lining the outer side of the hook (Raz and Ecker, 1999). It is possible, therefore, that endogenous PIF5 regulates the spatial expression of one or more enzymes involved in ethylene biosynthesis, mediating differential ethylene levels during the development of the apical hook.

Surprisingly, PIF5-OX seedlings grown in red light for 6 h or more have strikingly lower levels of phyB and phyC than wild-type seedlings (Figures 4B and 4C). The light-induced rapid degradation of phyB and phyC protein in PIF5-OX seedlings is sensitive to MG132 treatment and hence appears to be mediated by the ubiquitin-proteasome pathway (Figure 4). Furthermore, in PIF5-OX (mAPB) seedlings, phyB protein levels are unaffected (Figure 6; see Supplemental Figure 6 online). Collectively, these data show that overexpression of PIF5 with a functional APB motif, capable of interaction with phyB (Pfr), causes a decline in phyB (and phyC, which is stabilized by phyB) levels. There are at least two possible explanations for this observation: either the overexpression of PIF5 results in an altered physiological state that favors phytochrome degradation, or the loss of phyB is a direct consequence of the interaction of phyB with overexpressed PIF5, followed by degradation via the 26S proteasome pathway. The data presented in this study support the latter hypothesis. It is unlikely that changes in ethylene levels are responsible for the reduced phyB levels in PIF5-OX seedlings. The seedlings affected in ethylene signaling, *ctr1* and *ein2*,

respond normally to red light (Figure 1F). Furthermore, all seedlings treated with ACC have short hypocotyls in red light, including *phyB* mutant seedlings (Figure 2B), indicating either that ACC causes its effects on hypocotyl elongation in a manner unrelated to phyB or that its effects are downstream of phyB. Finally, the non-phyB binding mutant protein, PIF5-OX (mAPB), retains the capacity to elicit the triple response in dark-grown seedlings but lacks the capacity to induce hyposensitivity to prolonged Rc, clearly differentiating between these two molecular effects of the overexpressed transcription factor. Thus, changes in phyB protein levels in PIF5-OX and *pif5* mutant seedlings are more likely due to changes in PIF5 protein levels in these seedlings and require direct interaction with the photoreceptor (Figures 4 and 6; see Supplemental Figures 6 and 7 online).

The changes observed in phyB levels are sufficient to cause the observed phenotypes in the light. As shown by Wester et al. (1994), a 1.2-fold increase in phyB protein levels caused a change of 1 mm or more in hypocotyl length. The *pif5-2* mutants are hypersensitive to prolonged Rc, with a >1-mm difference in hypocotyl length from the wild type, and have 1.6-fold elevated phyB levels (Figures 1, 4, and 5). Conversely, PIF5-OX seedlings are hyposensitive to prolonged Rc, have dramatically reduced phyB levels within 3 to 6 h of growth in Rc (Figures 4B and 4F), and have reduced phyB levels after 3 d in Rc (Figure 5; see Supplemental Figure 7 online) compared with wild-type seedlings. Seedlings overexpressing PIF5 appear similar to *phyB*-null seedlings after 3 d in red light, and this response is dependent upon saturating red fluence rates (Figure 1). We hypothesize that saturating intensities of red light (>0.1 $\mu mol \cdot m^{-2} \cdot s^{-1}$) result in the maximum photoactivation of phyB, causing more phyB protein to be sequestered by the increased APB activity from overexpressed PIF5, resulting in the observed phenotype in prolonged red light. Consistent with this hypothesis, PIF5-OX seedlings growing in red light intensities of 1 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and higher exhibit phenotypes similar to those of *phyB* mutant seedlings and etiolated wild-type seedlings (Figure 1).

The data presented here indicate that endogenous PIF5 reduces phyB levels in prolonged Rc and, therefore, is indirectly responsible for the lower sensitivity of the wild-type hypocotyl compared with the hypersensitive *pif5* mutant, which accumulates higher phyB protein levels under these conditions. Interestingly, PIF5-OX seedlings accumulate higher phyA levels in the dark (Figure 4A; see Supplemental Figures 6A and 7D online). It will be interesting to test whether the elevated ethylene levels in dark-grown PIF5-OX seedlings somehow cause the higher accumulation of phyA protein in these seedlings. It can be speculated that elevated ethylene levels may cause changes in phyA accumulation by altering physiological or biochemical processes in the dark. We did not observe higher phyA levels in PIF5-OX seedlings exposed to prolonged red light irradiation (see Supplemental Figure 7A online). With regard to hypocotyl length, the effects of PIF5-OX on phyB appear to override any residual phyA effect that might remain in prolonged light.

Previous studies with *pif3* and *pif4* mutants have reported hypersensitive phenotypes in red light (Huq and Quail, 2002; Kim et al., 2003). Monte et al. (2004) reported that *pif3* mutant seedlings had slightly higher (1.5- to 2-fold) phyB levels compared with the wild type when grown in prolonged Rc and suggested that the hypersensitive phenotype of these mutants could be the result of these higher levels of phyB. However, the phyB levels were not rigorously quantitated, nor were the apparent differences statistically analyzed. Here, we show that the PIF5-OX and *pif5* mutant seedlings have statistically significant reciprocal differences in levels of phyB, which consequently appear to cause the observed phenotypes in prolonged red light. Most importantly, we show that these changes in phyB levels are dependent upon APB activity, indicating that direct interaction between the photoreceptor and transcription factor molecules is necessary for the phyB degradation observed. Recent studies with other *pif* mutants suggest that this may be a common phenomenon and that the PIF proteins may cause phenotypes in prolonged red light indirectly by changing phyB photoreceptor levels (P. Leivar-Rico and P.H. Quail, unpublished data; B. Al-Sady and P.H. Quail, unpublished data), rather than by functioning directly as phyB signaling intermediates in the transduction pathway, as has been widely assumed. It will be of interest to examine the role of the APB motif in these PIF proteins with regard to changes in phy protein levels and whether this is a result of codegradation.

METHODS

Plant Material, Growth Conditions, and Measurements

T-DNA insertional disruption mutants were identified in *Arabidopsis thaliana* ecotype Col by searching the SIGnAL database (Alonso et al., 2003) and obtained from the ABRC: *pif5-1* (Salk_143659), *pif5-2*, (Salk_072306), and *pif5-3* (*pil6-1*; Salk_087012). Homozygous lines were isolated using gene-specific and T-DNA–based primers for PCR analysis as described (Khanna et al., 2006). The mutants were crossed twice with Col (wild type), and wild-type siblings were maintained for phenotypic comparisons. Phenotypes were further confirmed by comparison with Col. *Arabidopsis* (Col) plants were transformed with PIF5 cDNA driven by the 35S promoter (35S::PIF5) to obtain PIF5-OX lines. Two independent lines, PIF5-OX (1-5) and PIF5-OX (22-1), were used in this study. PIF5-OX (1-5) was used in experiments for which the line is not specified. Site-directed mutagenesis was performed using the Quick Change site-directed mutagenesis kit (Stratagene) to create mutant APB (G37A) lines [PIF5-OX (mAPB)].

Seedlings were grown on plates containing growth medium without sucrose (Hoecker et al., 1999). Some of the plates contained different concentrations of $AgNO_3$ or ACC added to the growth medium (as indicated). Red light and FRc treatments were performed as described (Wagner et al., 1991). Fluence rates were measured using a spectroradiometer (model L1-1800; Li-Cor). Hypocotyl length, hook angles, and cotyledon angles were measured using digital images taken with a Nikon Coolpix 990 digital camera and NIH Imaging software (National Institutes of Health). Approximately 30 seedlings of each genotype were used for each analysis. SE values were calculated from three biological replicates.

RNA Isolation and Analysis

Total RNA was extracted using the RNeasy Plus plant mini kit according to the manufacturer's recommendations (Qiagen). For RT-PCR, SuperScript III RNase H^{-} reverse transcriptase (Invitrogen) was used to synthesize the first strand of cDNA with the oligo(dT20) primer (Invitrogen) from 1 μg of total RNA. cDNA was diluted four times with water, and 1 μL was used for real-time PCR (MyIQ single-color real-time PCR detection system; Bio-Rad). Eva-green (Biotium) was used for detection according

to the manufacturer's recommendations, except that final concentrations of 0.1% Tween 20, 0.1 mg/mL BSA, and 5% DMSO were also added to the PCR mix. The gene *At2g32170* was used as a normalization control (Czechowski et al., 2005). The expression of this gene does not change in darkness or red light (data not shown). Each PCR was repeated twice. Gene expression data were represented relative to the average value for the wild type grown in darkness in each experiment, after normalization to the control. All experiments were performed with three independent biological replicates.

Measurements of Ethylene Production

Ethylene production was determined in 4-d-old etiolated seedlings or in 4-d-old red light–grown seedlings. One hundred etiolated seedlings or 50 red light–grown seedlings were placed in 4-mL vials, and ethylene accumulation was determined after 24 h of incubation in the dark or red light at 25°C. The ethylene concentration in the headspace of the vials was determined by gas chromatography (5890A Hewlett-Packard gas chromatograph).

Protein Extraction and Immunoblot Analysis

For the detection of phyA, phyB, and phyC proteins, 4-d-old seedlings were used for protein extraction in 1:2 (w/v) heated (in a boiling water bath) denaturing extraction buffer (100 mM Tris-HCl, pH 7.8, 4 M urea, 5% SDS, 15% glycerol, and 10 mM DTT) with protease inhibitors (2 μg/L aprotinin, 3 μg/L leupeptin, 1 μg/L pepstatin, 2 mM phenylmethylsulfonyl fluoride, and 30 μL protease inhibitor cocktail [Sigma-Aldrich]) per milliliter of plant extract. Plant extracts were boiled for 5 min and cleared by centrifugation at 15,000*g* for 15 min. Thirty to 100 μg of total protein was separated on 10% SDS-PAGE gels, and blots were probed with specific anti-phy antibodies as described. For the detection of phyD and phyE, seedlings were extracted using sequential $(NH_4)_2SO_4$ fractionation as described (Hirschfeld et al., 1998). An equivalent of 200 μg of total protein [before $(NH_4)_2SO_4$ fractionation] was applied for each immunoblot analysis. As described, some seedlings were treated with 30 μM MG132 (a 26S proteasome inhibitor) for 5 h before protein extraction.

Detection, Quantification, and Analysis of phyB Protein Levels in 4-d-Old Dark-Grown or Rc-Grown Seedlings

Col wild-type, *pif5-2* mutant, and PIF5-OX seedlings were grown either in the dark for 4 d or under Rc (7 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 3 d following a 21-h dark period. Total protein was extracted into 1:2 (w/v) heated denaturing extraction buffer as described above. Protein concentration was determined with the RC-DC protocol (Bio-Rad).

Total protein was separated on 10% SDS-PAGE gels, transferred to Immobilon membranes (Millipore), and probed with either anti-phyB or anti-tubulin (Sigma-Aldrich) antibodies. To compare phyB protein levels among different genotypes of Rc-grown seedlings, 5 μg of total protein of each genotype, together with a dilution series of phyB protein standards, was analyzed by immunoblots. Plant extract of Col wild type (Rc) was used for the dilution series: 7.5, 5, 2.5, 1.25, and 0.625 μg was loaded, representing 150, 100, 50, 25, and 12.5% of wild-type levels (see Supplemental Figures 7B and 7C online). For the phyA protein standard, twice as much extract was used to generate a dilution series from 300 to 25% (see Supplemental Figure 7B online, top panel). Enhanced chemiluminescence (Amersham) signals from each blot were recorded on x-ray film with multiple exposures and analyzed using NIH Image software. The linearity between enhanced chemiluminescence signals of each band and the corresponding phyB protein standards was analyzed in each exposure, and the best linear fit was chosen to calculate relative phyB protein levels for each genotype. The unit of phyB protein of each genotype was determined as a percentage of Col wild type (Rc). The same approach was used to analyze relative tubulin protein levels in each genotype compared with tubulin standards produced by Col wild type (Rc). The unit of tubulin protein level was labeled as the percentage of Col wild type (Rc).

The final concentration of phyB protein for each genotype was expressed as phyB/tubulin, labeled as the percentage of Col wild type (Rc). Mean values of phyB protein level (phyB/tubulin) of each genotype and SD were calculated from three biological replicates. As an internal control, phyB protein levels among different genotypes of dark-grown seedlings were also calculated using the same method.

Accession Number

Sequence data for PIF5 can be found in the Arabidopsis Genome Initiative database under accession number At3g59060.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Relative *PIF5* Transcript Levels in *pif5* Mutant and PIF5-OX Seedlings Grown in the Dark or Exposed to Rc.

Supplemental Figure 2. *ACS2*, *ACS6*, and *ACS11* Transcript Levels in Col Seedlings Treated with Silver or ACC and Compared with Levels in *pif5-2* Mutant and PIF5-OX Seedlings.

Supplemental Figure 3. Comparison of phyD and phyE Protein Levels between Col and PIF5-OX Seedlings.

Supplemental Figure 4. Treatment with MG132, an Inhibitor of the 26S Proteasome Pathway, Slows Phytochrome Protein Degradation in Rc.

Supplemental Figure 5. Analysis of *PHY* Transcript Levels in *pif5* Mutant and PIF5-OX Seedlings Grown in the Dark or in Red Light.

Supplemental Figure 6. Comparison of phyA and phyB Protein Levels in Seedlings Overexpressing PIF5 with or without a Functional APB Motif.

Supplemental Figure 7. Quantification and Analysis of phy Protein Levels in Seedlings Grown in the Dark or in Rc.

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