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Arabidopsis Transcription Factor ELONGATED HYPOCOTYL5 Plays a Role in the Feedback Regulation of Phytochrome A Signaling [C] [W]

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Phytochrome A (phyA) is the primary photoreceptor responsible for perceiving and mediating various responses to far-red light in *Arabidopsis thaliana*. FAR-RED ELONGATED HYPOCOTYL1 (FHY1) and its homolog FHY1-LIKE (FHL) are two small plant-specific proteins essential for light-regulated phyA nuclear accumulation and subsequent phyA signaling processes. FHY3 and its homolog FAR-RED IMPAIRED RESPONSE1 (FAR1) are two transposase-derived transcription factors that directly activate *FHY1*/*FHL* transcription and thus mediate subsequent phyA nuclear accumulation and responses. Here, we report that ELONGATED HYPOCOTYL5 (HY5), a well-characterized bZIP transcription factor involved in promoting photomorphogenesis, directly binds ACGT-containing elements a few base pairs away from the FHY3/FAR1 binding sites in the *FHY1*/*FHL* promoters. We demonstrate that HY5 physically interacts with FHY3/FAR1 through their respective DNA binding domains and negatively regulates FHY3/FAR1-activated *FHY1*/*FHL* expression under far-red light. Together, our data show that HY5 plays a role in negative feedback regulation of phyA signaling by attenuating FHY3/FAR1-activated *FHY1*/*FHL* expression, providing a mechanism for fine-tuning phyA signaling homeostasis.

INTRODUCTION

Phytochromes are red (R)/far-red (FR) light photoreceptors that play fundamental roles in photoperception of the light environment and the subsequent adaptation of plant growth and development (Quail et al., 1995; Whitelam and Devlin, 1997; Wang and Deng, 2003; Bae and Choi, 2008). There are five distinct phytochromes in *Arabidopsis thaliana*, designated phytochrome A (phyA) to phyE. PhyA is light-labile and is the primary photoreceptor responsible for perceiving and mediating various responses to FR light, whereas phyB-phyE are light-stable, and phyB is the predominant phytochrome regulating responses to R light (Sharrock and Quail, 1989; Somers et al., 1991; Nagatani et al., 1993; Parks and Quail, 1993; Reed et al., 1993; Whitelam et al., 1993). Phytochromes are synthesized in the cytosol in their inactive Pr form. Upon light irradiation, phytochromes are converted from the R light–absorbing Pr forms to the FR light–absorbing Pfr forms, and the Pfr forms of phytochromes (which are generally considered to be the biologically active forms) are translocated from the cytosol into the nucleus, triggering a signaling cascade that alters the expression of target genes and ultimately leads to the modulation of the biological responses (Sakamoto and Nagatani, 1996; Kircher et al., 2002; Quail, 2002; Jiao et al., 2007). Thus, light-regulated translocation of the photoreceptors from the cytosol into the nucleus is a key event in the phytochrome signaling cascade.

PhyA nuclear import is rapid and can be induced by either FR or R light; however, phyB nuclear import is relatively slow, only occurs in R light, and can be reversed by FR light (Kircher et al., 1999; Nagatani, 2004; Kevei et al., 2007; Fankhauser and Chen, 2008). The C-terminal half of phyB contains a putative nuclear localization signal (NLS), which is masked by the N-terminal half in darkness. Light triggers a conformational change in phyB, potentially unmasking the NLS and thus allowing its nuclear import (Chen et al., 2005; Fankhauser and Chen, 2008). By contrast, phyA does not contain any known NLS. Therefore, phyA translocation appears to depend on other components. Recently, it has been shown that two small plant-specific proteins, FAR-RED ELONGATED HYPOCOTYL1 (FHY1) and its homolog FHY1-LIKE (FHL), are essential for nuclear accumulation of light-activated phyA and subsequent light responses (Hiltbrunner et al., 2005, 2006; Rösler et al., 2007). A database search for FHY1/FHL homologs identified FHY1-like proteins in numerous plant species, and the only motifs conserved among

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these FHY1-like proteins are the NLS in their N-terminal region and the phyA binding domain at the C terminus, suggesting that the phyA nuclear import mechanism discovered in *Arabidopsis* might be conserved in higher plants (Genoud et al., 2008). Two transposase-derived transcription factors, FHY3 and FAR-RED IMPAIRED RESPONSE1 (FAR1), act together to activate directly the transcription of *FHY1* and *FHL*, thus indirectly regulating phyA nuclear accumulation and phyA responses (Lin et al., 2007, 2008).

Genetic and molecular studies have led to the identification of numerous signaling intermediates that are either specific for individual photoreceptors or shared by multiple types of photoreceptors. Many of these intermediates are transcription factors or transcriptional regulators (for reviews, see Quail, 2002; Wang and Deng, 2003; Jiao et al., 2007). In addition to FHY3 and FAR1, a group of basic helix-loop-helix class transcription factors, also known as PHYTOCHROME INTERACTING FACTORS (PIFs), including PIF1, PIF3, PIF4, and PIF5, have been shown to bind photoactivated phytochromes directly and play central roles in phytochrome signaling networks (Ni et al., 1998; Huq and Quail, 2002; Kim et al., 2003; Huq et al., 2004; Khanna et al., 2004; Duek and Fankhauser, 2005; Castillon et al., 2007). Recent data showed that these PIF proteins accumulate in dark-grown seedlings and together act as constitutive repressors of photomorphogenesis, while upon light exposure, photoactivated phytochromes induce rapid phosphorylation and degradation of these transcription factors, allowing photomorphogenesis to begin (Bauer et al., 2004; Al-Sady et al., 2006; Shen et al., 2007; Leivar et al., 2008; Lorrain et al., 2008; Shen et al., 2008). ELONGATED HYPOCOTYL5 (HY5), a constitutively nuclear bZIP transcription factor, has been shown to function as a positive regulator of photomorphogenic development under a wide spectrum of wavelengths, including FR, R, blue (B), and UV-B, by binding directly to the promoters of a large number of light-responsive genes in vivo (Koornneef et al., 1980; Oyama, et al., 1997; Osterlund et al., 2000; Ulm et al., 2004; Lee et al., 2007). Multiple photoreceptors, including phytochromes and cryptochromes, promote the accumulation of HY5 under specific light conditions, possibly by reducing the nuclear abundance of CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1), an E3 ubiquitin ligase targeting HY5 for proteasome-mediated degradation in darkness (Osterlund and Deng, 1998; Osterlund et al., 2000). LONG HYPOCOTYL IN FAR-RED1 (HFR1) and LONG AFTER FAR-RED LIGHT1 (LAF1), atypical basic helix-loop-helix and R2R3-MYB transcription factors, respectively, were identified as positive regulators of phyA signaling (Fairchild et al., 2000; Fankhauser and Chory, 2000; Soh et al., 2000; Ballesteros, et al., 2001). Recently, it was shown that these two transcription factors interact with each other, and this interaction stabilizes both factors and enhances phyA photoresponses (Jang et al., 2007).

Previous studies showed that *FHY1*/*FHL* transcript levels were rapidly downregulated when dark-grown plants were exposed to FR light (Desnos et al., 2001; Lin et al., 2007), indicating that *FHY1*/*FHL* expression is subject to strict negative feedback regulation by phyA signaling, which is, however, poorly understood. Here, we show that the bZIP transcription factor HY5 acts in the feedback downregulation of *FHY1*/*FHL* expression by phyA signaling. We demonstrate that HY5 directly binds ACGT-containing elements (ACEs) in close proximity to the FHY3/FAR1 binding sites in the *FHY1*/*FHL* promoters. HY5 physically interacts with FHY3 and FAR1 and interferes with FHY3/FAR1 binding to the *FHY1*/*FHL* promoters. Thus, HY5 negatively regulates *FHY1*/*FHL* expression upon FR light exposure by modulating the activities of the transcriptional activators FHY3 and FAR1. Therefore, HY5 repression of *FHY1*/*FHL* expression likely represents a key mechanism in the feedback regulation of phyA signaling.

RESULTS

HY5 Directly Binds to the *FHY1*/*FHL* Promoters

To investigate whether other transcription factors are involved in regulating *FHY1*/*FHL* expression, we performed yeast one-hybrid assays to test whether PIF3, HY5, LAF1, and HFR1, four transcription factors known to act in the phytochrome signaling pathways, bind to the *FHY1* and *FHL* promoters. Our results show that of these four proteins, only HY5 binds directly to both promoters (FHY3 and FAR1 were included as positive controls) (Figure 1A), suggesting that HY5 may play a role in regulating *FHY1*/*FHL* expression.

As previous research indicated that HY5 binds ACEs (Chattopadhyay et al., 1998; Lee et al., 2007; Shin et al., 2007), we analyzed the distribution of ACEs in the *FHY1* and *FHL* promoters. Interestingly, both promoters were found to harbor two short regions that contain ACE elements: one region very close to the stop codons of their respective upstream genes and the other region located ~150 to 300 bp upstream of their respective ATG start codons (Figure 1B). We then divided the *FHY1*/*FHL* promoters into four overlapping fragments, designated A, B, C, and D, in which the B and D fragments correspond to the two ACE-containing regions (Figure 1B), and generated yeast one-hybrid reporter constructs allowing the respective fragments to drive *LacZ* reporter gene expression. As expected, HY5 does not bind to the A or C fragments of either promoter, consistent with the fact that there are no ACEs in these fragments. Although HY5 weakly binds to the D fragment of the *FHY1* promoter, it does not bind to the corresponding fragment of the *FHL* promoter (Figure 1C). By contrast, HY5 binds strongly to the B fragments of both promoters (Figure 1C), suggesting that the B fragments contain the major HY5 binding sites. As HY5 has a close homolog in the *Arabidopsis* genome, known as HY5 HOMOLOG (HYH), we tested whether HYH also binds to the *FHY1* promoter. However, HYH failed to bind either the full-length or the B fragment of the *FHY1* promoter (see Supplemental Figure 1 online), indicating that these two homologs target diverse genes. Interestingly, as the B fragments also contain FHY3/FAR1 binding sites (FBSs; Lin et al., 2007), FHY3 and FAR1 are able to bind to the B fragments of both promoters as well (Figure 1C). Collectively, our data show that HY5, FHY3, and FAR1 bind to the same fragments of the *FHY1*/*FHL* promoters.

A recent study using the chromatin immunoprecipitation (ChIP)-chip approach identified 3894 putative HY5 binding targets in the *Arabidopsis* genome (Lee et al., 2007), among which, *FHY1* was listed as a potential HY5 binding target (see

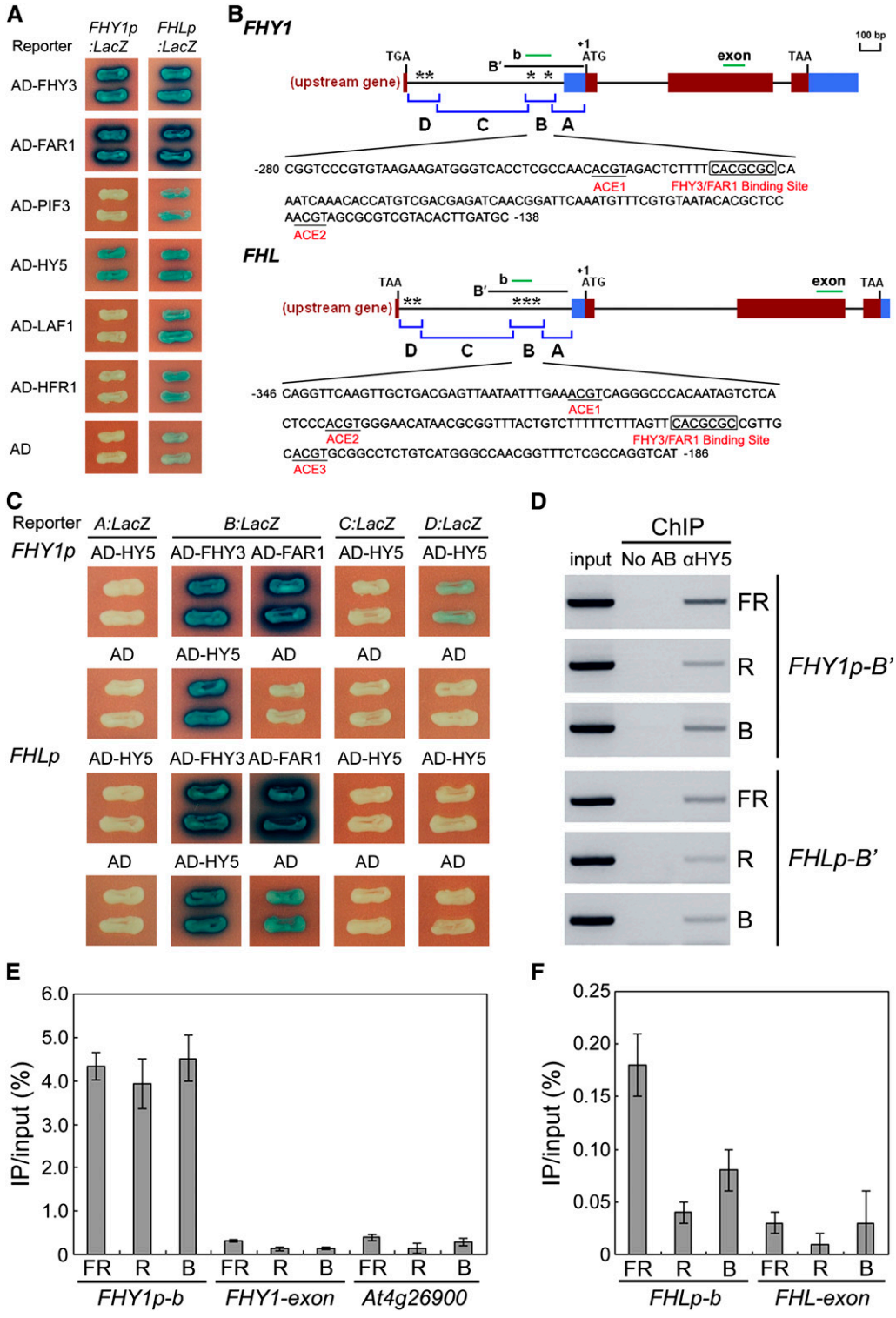

Figure 1. Transcription Factor HY5 Binds to the *FHY1* and *FHL* Promoters in Yeast Cells and in Vivo.

(A) Yeast one-hybrid assays to test whether transcription factors PIF3, HY5, LAF1, and HFR1 bind to the *FHY1* and *FHL* promoters. FHY3 and FAR1 were included as positive controls and empty vector expressing AD domain alone as negative controls. *LacZ* reporter gene expression (leading to blue color on the plates containing X-gal) was driven by the *FHY1* or *FHL* promoter in yeast.

(B) Diagram of the promoter fragments of *FHY1* and *FHL* and the sequences of B fragments of the *FHY1* and *FHL* promoters. The exon-intron structure

Supplemental Table 1 in Lee et al., 2007). To confirm this data experimentally and to examine whether HY5 binds to the *FHL* promoter in vivo, we performed ChIP assays using wild-type *Arabidopsis* seedlings. As HY5 protein accumulates mostly in light conditions (Osterlund et al., 2000; see Supplemental Figure 2 online), we then examined whether HY5 binds to the *FHY1*/*FHL* promoters in continuous FR, R, and B light. Our multiplex PCR and quantitative PCR (qPCR) both show that HY5 specifically binds to the *FHY1* promoter in vivo in all three monochromatic light conditions (Figures 1D and 1E), compared with an exon fragment of *FHY1* and the 3′ untranslated region of *At4g26900*, a negative control used in the previous ChIP-chip study. However, although our results also show that HY5 binds to the *FHL* promoter (Figures 1D and 1F), this binding seems much weaker than that to *FHY1* as our qPCR data show that the recovery rate of an *FHL* promoter fragment in the FR ChIP sample is ~25-fold less than that of an *FHY1* promoter fragment. Moreover, at least for the *FHL* promoter fragment, we observed a higher recovery rate in the FR sample than in the R and B samples (Figures 1D and 1F). Taken together, we conclude that HY5 binds to the *FHY1* and *FHL* promoters in yeast cells and in vivo.

HY5 Binds the ACEs Closest to FBSs in the *FHY1*/*FHL* Promoters

We next performed yeast one-hybrid assays to delineate the exact ACEs that are bound by HY5 in the B fragments of the *FHY1*/*FHL* promoters. As there are two and three ACEs in the B fragments of the *FHY1* and *FHL* promoters, respectively, a total of 10 reporter constructs were generated to allow the respective wild-type subfragments and their corresponding mutants (mut; in which the respective ACGT was mutated to tttT) to drive *LacZ* reporter gene expression in yeast cells (Figure 2A). Our results show that for the two ACEs in the *FHY1* promoter, HY5 binds the wild-type ACE1, but not its mutant, nor ACE2 in yeast cells (Figure 2B). We also mutated ACE1 and ACE2 in the context of the full B fragment of the *FHY1* promoter to determine which ACE is the preferred binding site for HY5. Again, mutation of ACE1 almost abolished binding of HY5 to this promoter fragment, whereas mutation of ACE2 did not affect binding, confirming that HY5 primarily binds ACE1 in the B fragment of the *FHY1* promoter (Figure 2C).

For the three ACEs in the B fragment of the *FHL* promoter, our data show that HY5 does not bind to the wild-type subfragments of ACE1 or ACE2 (Figure 2B). However, for unknown reasons, the subfragments containing either wild-type or mutant ACE3 always induced strong *LacZ* reporter gene expression in yeast cells (Figure 2B). We therefore performed an electrophoretic mobility shift assay (EMSA) to test whether HY5 binds to the ACE3 subfragment in vitro. The ACE1 subfragment of the *FHY1* promoter was also included in the assay as a positive control. Our data show that the GST-HY5 fusion protein (glutathione *S*-transferase fused with HY5), but not GST alone, bound the ACE1 subfragment of the *FHY1* promoter and the ACE3 subfragment of the *FHL* promoter in vitro (Figure 2D), indicating that ACE3 is the HY5 binding site in the B fragment of the *FHL* promoter.

Interestingly, we noticed that ACE1 of the *FHY1* promoter, and ACE3 of the *FHL* promoter, are the respective ACEs that are closest to FBSs in the *FHY1*/*FHL* promoters, and there are only 10 bp or less between these two *cis*-elements (Figure 1B). We therefore conclude that HY5 binds the ACEs closest to the FHY3/FAR1 binding sites in the *FHY1* and *FHL* promoters.

HY5 Interacts with FHY3 and FAR1

As HY5 and FHY3/FAR1 bind *cis*-elements that are so close to each other in the *FHY1*/*FHL* promoters, it was intriguing to investigate whether these two types of transcription factors could physically interact with each other. To this end, we first performed yeast two-hybrid assays using bait vectors expressing either the full-length, N-terminal (harboring the COP1 interaction motif), or C-terminal (encoding the bZIP domain) domains of HY5 fused to the LexA DNA binding domain and prey vectors fusing the activation domain (AD) to the indicated *Arabidopsis* proteins (Figures 3A and 3B). Our data show that the full-length HY5 protein indeed interacts with FHY3 and FAR1, and the bZIP domain of HY5 is responsible for this interaction (AD-COP1 and AD-HY5 were included as positive controls for the N- and C-terminal domains of HY5, respectively) (Figure 3B).

Figure 1. (continued).

of *FHL* was based on the fact that *FHL* has an alternatively spliced transcript consisting of three exons and encoding a 201–amino acid isoform (GenBank accession number HM029245), in addition to the reported 181–amino acid FHL protein (Zeidler et al., 2001; Zhou et al., 2005). The genes upstream of *FHY1* and *FHL* are *At2g37680* and *At5g02190*, respectively. The adenine residue of the respective translational start codon (ATG) was assigned position +1, and the numbers flanking the sequences of the B fragments were counted based on this number. Asterisks indicate ACEs. A, B, C, and D indicate the corresponding promoter fragments used in yeast one-hybrid assays shown in **(C)**. The black lines indicate the regions amplified by ChIP-PCR shown in **(D)**. The short green lines depict the location of amplicons used for ChIP-qPCR shown in **(E)** and **(F)**.

(C) Yeast one-hybrid assays showing that HY5 binds to the B fragments of the *FHY1* and *FHL* promoters. FHY3 and FAR1 were included as positive controls, and empty vector expressing AD domain alone as negative controls.

(D) Representative results of the ChIP-PCR assays. Chromatin fragments (~500 bp) were prepared from 4-d-old wild-type *Arabidopsis* seedlings grown under continuous FR, R, and B light conditions, immunoprecipitated by the polyclonal HY5 antibodies, and the precipitated DNA PCR-amplified using the primer pairs indicated. Input, PCR reactions using the samples before immunoprecipitation. AB, antibody.

(E) and **(F)** qPCR analysis of the promoter (b fragments) and exon fragments of *FHY1* and *FHL* in anti-HY5 ChIP assays. The 3′ untranslated region of *At4g26900*, a negative control used in a previous study (Lee et al., 2007), was used as a negative control in these assays. The ChIP values were normalized to their respective DNA inputs. Error bars represent SD of triplicate experiments.

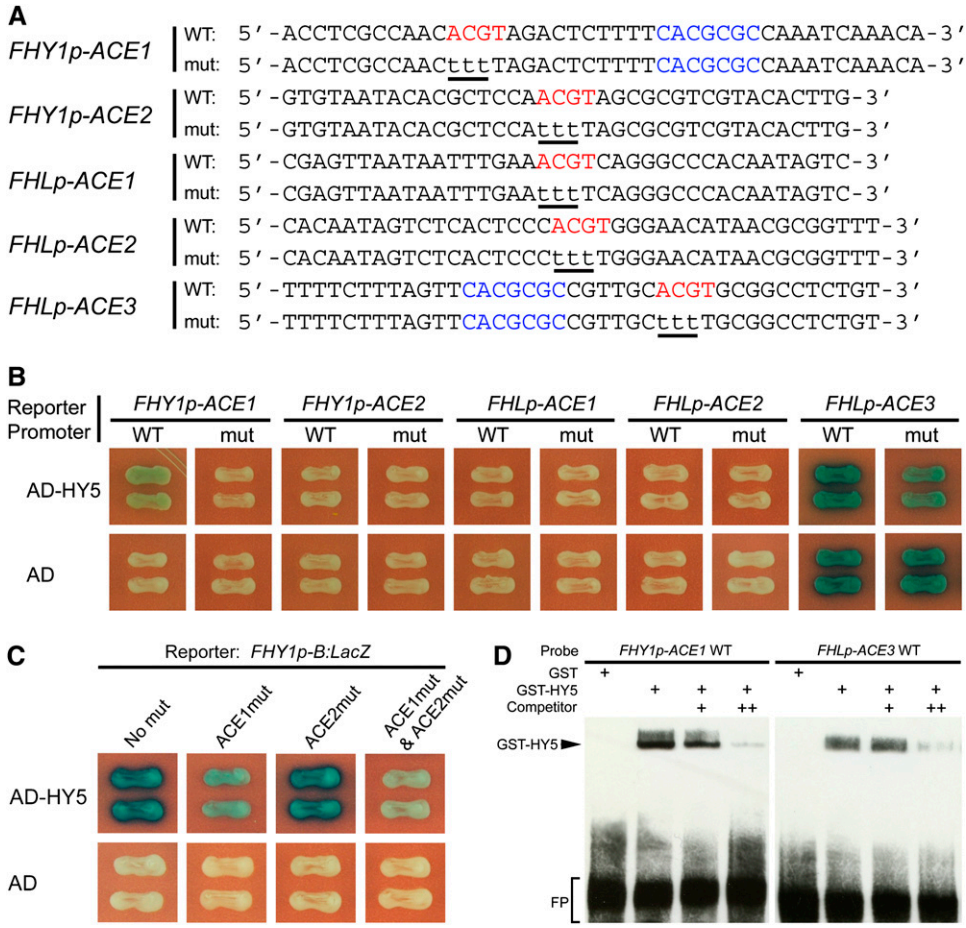

Figure 2. HY5 Binds the ACEs Closest to the FHY3/FAR1 Binding Sites in the *FHY1* and *FHL* Promoters.

(A) Diagram of the wild-type (WT) and mutant (mut) *FHY1* and *FHL* subfragments used to drive *LacZ* reporter gene expression in yeast one-hybrid assays and as EMSA probes. Wild-type ACE elements are shown in red, and FBS motifs are shown in blue. Nucleotide substitutions in the mutant fragments are underlined.

(B) Yeast one-hybrid assays showing that HY5 binds the wild-type ACE1 of the *FHY1* promoter but not ACE2 of the *FHY1* promoter, or ACE1 or ACE2 of the *FHL* promoter.

(C) Yeast one-hybrid assays showing that ACE1 is the primary binding site for HY5 in the B fragment of the *FHY1* promoter. The B fragment of the *FHY1* promoter (sequence shown in Figure 1B) was mutated to abolish ACE1, ACE2, or both and used to drive *LacZ* reporter gene expression. In these assays, the respective ACGT was mutated to AaaT to facilitate mutagenesis reactions.

(D) EMSAs showing that GST-HY5 protein, but not GST by itself, specifically binds to the wild-type *FHY1p-ACE1* and *FHLp-ACE3* probes. The respective unlabeled probes (with the same sequence as the biotin-labeled probes) were used as competitors. FP, free probe.

FHY3 and FAR1 were reported to contain three domains: an N-terminal C2H2 zinc finger domain, a central putative core transposase domain, and a C-terminal SWIM zinc finger domain (Lin et al., 2008). To define which of these domains of FHY3 and FAR1 interact with HY5, we fused them individually with LexA as indicated (Figure 3C) and then used these fusions as bait proteins in yeast two-hybrid assays. Unexpectedly, the SWIM zinc finger domain of FAR1 showed strong *trans*-activation activity in yeast cells (Figure 3D). Nevertheless, FHY3N and FAR1N also showed interaction with AD-HY5, allowing the conclusion that the C2H2 zinc finger motifs (i.e., the DNA binding domains of FHY3 and FAR1) may be responsible for interacting with HY5 (Figure 3D). To confirm this interaction further, we performed in vitro pull-down assays using GST-tagged HY5 and 6×His-tagged N-terminal domains of FHY3 and FAR1. Our data showed that GST-HY5, but not GST alone, was able to pull down the N-terminal domains of FHY3 and FAR1 (Figure 3E). Taken together, these data show that HY5 interacts with FHY3 and FAR1, and their DNA binding domains mediate their interactions.

To substantiate the physical interaction between HY5 and FHY3 in planta, we conducted firefly luciferase complementation imaging (LCI) assays (Chen et al., 2008) by transiently expressing HY5-NLuc and CLuc-FHY3N fusions in *Nicotiana benthamiana* leaf cells. As shown in Figure 3F, coexpression of HY5-NLuc and CLuc-FHY3N led to strong LUC activity. By contrast, HY5-NLuc or CLuc-FHY3N cotransformed with control

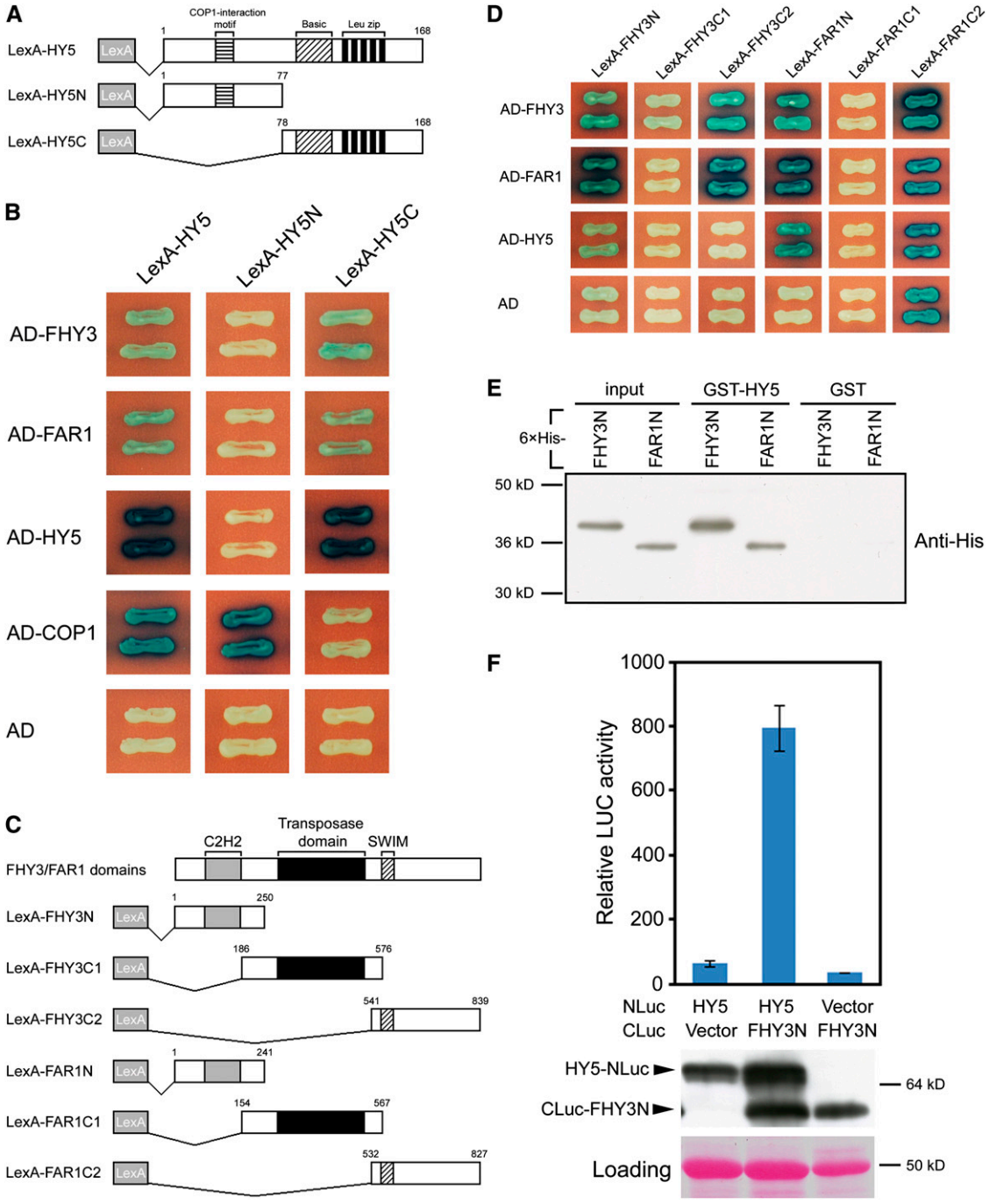

Figure 3. HY5 Interacts with FHY3 and FAR1.

(A) Schematic diagram of bait proteins (HY5, HY5N, and HY5C fused with LexA DNA binding domains).
(B) HY5C (corresponding to the bZIP domain of HY5) interacts with FHY3 and FAR1 in yeast cells. AD-COP1 and AD-HY5 were included as positive controls for interactions between HY5N and COP1 and the dimerization of bZIP domains, respectively.
(C) Schematic diagram of bait proteins (FHY3N, FHY3C1, FHY3C2, FAR1N, FAR1C1, and FAR1C2 fused with LexA DNA binding domains).
(D) Yeast two-hybrid assays showing that the DNA binding domains of FHY3 and FAR1 (FHY3N and FAR1N) interact with HY5.
(E) In vitro pull down of FHY3N and FAR1N with HY5. The 6×His-tagged N-terminal fragments of FHY3 and FAR1 pulled down with GST-HY5 or GST were detected by anti-His antibody. Input, 5% of the purified 6×His-tagged target proteins used in pull-down assays.
(F) Luciferase complementation imaging assays showing that HY5 interacts with FHY3N in plant cells. Tobacco leaves were transformed with the construct pairs HY5-NLuc/CLuc, HY5-NLuc/CLuc-FHY3N, and NLuc/CLuc-FHY3N. The leaves were observed for fluorescence imaging 3 d after the

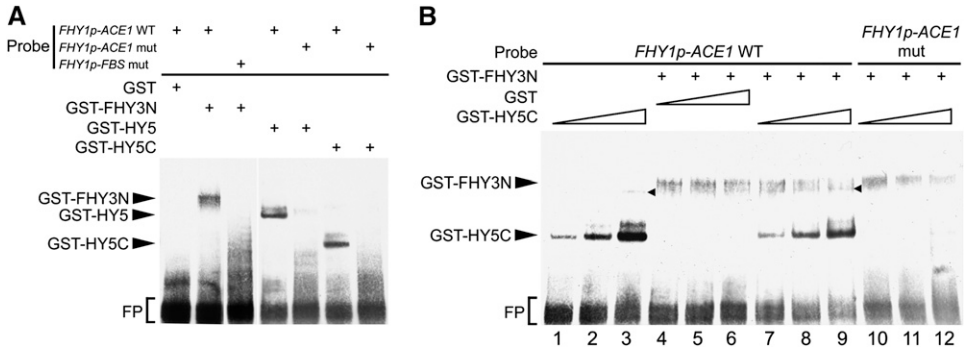

Figure 4. HY5 Interferes with FHY3 for Binding to the *FHY1* Promoter.

(A) GST-FHY3N, GST-HY5, and GST-HY5C proteins, but not GST by itself, bind to the wild-type (WT) *FHY1* promoter but not to the FBS (GST-FHY3N) and ACE (GST-HY5 and GST-HY5C) mutant (mut) probes. The sequences of *FHY1p-ACE1* wild-type and mutant probes are shown in Figure 2A. The sequence of *FHY1p-FBS* mut probe is identical to that of the *FHY1p-ACE1* wild-type probe except that the FBS motif (CACGCGC) was mutated to CAttttC. FP, free probe.

(B) Increasing amounts of GST-HY5C protein (lanes 7 to 9), but not GST (lanes 4 to 6), decrease the binding of GST-FHY3N to the wild-type *FHY1* promoter. Interaction between HY5C and FHY3N prevents GST-FHY3N from binding to the promoter (lanes 10 to 12). The triangles in lanes 3 and 9 indicate a polymer of HY5C bound to the probe.

vectors showed only background levels of LUC activity. These data support a physical interaction between HY5 and FHY3 in living plant cells.

HY5 Interferes with FHY3 for Binding to the *FHY1* Promoter

As the HY5 binding site is only a few base pairs away from the FHY3 binding site in the *FHY1* promoter, we next investigated how HY5 affects FHY3 binding to the *FHY1* promoter by EMSAs using fusion proteins expressed in *Escherichia coli*. For FHY3, we used GST-tagged FHY3N (the first 200 amino acids of FHY3) and validated its biochemical activity by showing that it binds to the wild type but not the FBS-mutated *FHY1* probe (Figure 4A). As GST-HY5 always migrates at a similar position as GST-FHY3N when they bind to the wild-type *FHY1* probes (Figure 4A), we used GST-tagged HY5C (the amino acids 78 to 168 of HY5) instead in our assays. GST-HY5C also binds to the wild type but not the ACE1-mutated *FHY1* probe and thus possesses the DNA binding activity of HY5 (Figure 4A).

We then examined how HY5 affects FHY3 binding to the *FHY1* promoter. As shown in Figure 4B, whereas increasing amounts of GST protein alone had no effect on GST-FHY3N binding to the promoter (lanes 4 to 6), increasing amounts of GST-HY5C protein obviously decreased the binding of GST-FHY3N to the wild-type *FHY1* promoter (lanes 7 to 9). Then, we investigated how the interaction between HY5 and FHY3 affects FHY3 binding to the promoter using the ACE1-mutated probe to which HY5 could not bind. Notably, increasing amounts of GST-HY5C protein dramatically decreased the binding of GST-FHY3N to the *FHY1* promoter even though GST-HY5C was not binding the probe (Figure 4B, lanes 10 to 12). Therefore, these data suggest that (1) increasing HY5 binding to the *FHY1* promoter simultaneously decreases FHY3 binding to the promoter, and (2) the physical interaction between HY5 and FHY3 prevents FHY3 from binding to the *FHY1* promoter.

HY5 Negatively Regulates FHY3/FAR1-Activated *FHY1*/*FHL* Transcription in Yeast and Plant Cells

To investigate how HY5 affects FHY3/FAR1-mediated *FHY1*/*FHL* transcription in yeast cells, we introduced another set of constructs into our yeast one-hybrid system. These pGAD-T7–based constructs were generated to express AD, HY5, or AD-HY5 fusion proteins in yeast cells (Figures 5A and 5B). As shown in Figure 5A, HY5 alone does not activate *LacZ* reporter gene expression, although it could bind to the *FHY1* promoter (cf. 1 and 2), consistent with the previous report that HY5 lacks the transcriptional activation domain and is unable to activate transcription in yeast cells (Ang et al., 1998). However, addition of an activation domain to HY5 (AD-HY5) allows HY5 to activate *LacZ* reporter gene expression (Figure 5A, cf. 2 and 3). As mentioned above (Figures 1C and 2B), the B fragment of the *FHL* promoter (possibly due to the ACE3-containing sequence shown in Figure 2A) induced strong background expression of the *LacZ* reporter gene (Figure 5B, 1 and 2).

We then examined how HY5 affects FHY3/FAR1-mediated reporter gene expression driven by the B fragments of the *FHY1*/*FHL* promoters. As shown in Figures 5A and 5B, AD-FHY3 and

Figure 3. (continued).

infiltration. The data shown are representative of three independent experiments, and error bars represent SD of four replicates. The middle panel shows an immunoblot for proteins isolated from tobacco leaves. Anti- full-length firefly LUC antibodies, which react with both the N- and C-terminal firefly LUC fragments, were used to detect the fusion proteins. The amount of protein loaded in each lane is indicated by Ponceau S staining of ribulose-1,5-bisphosphate carboxylase/oxygenase (bottom panel).

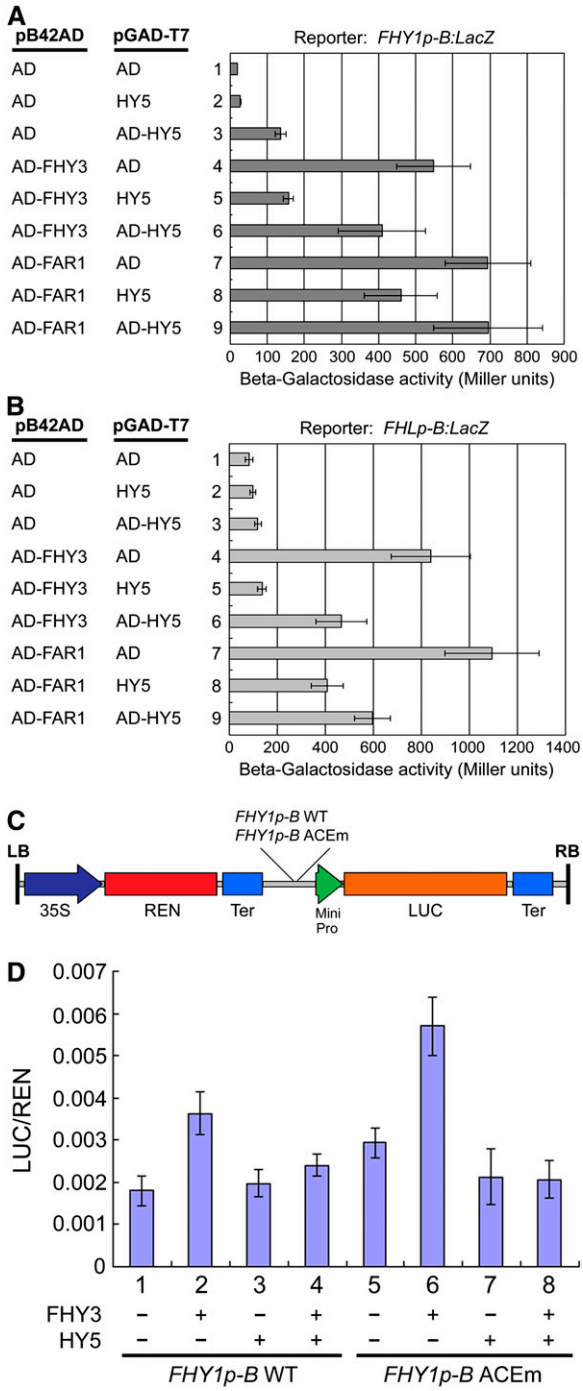

Figure 5. HY5 Negatively Regulates FHY3/FAR1-Activated *FHY1*/*FHL* Transcription in Yeast and Plant Cells.

(A) and **(B)** Quantification of β-galactosidase activity in yeast cells harboring the *FHY1p-B:LacZ* **(A)** or *FHLp-B:LacZ* **(B)** reporter construct and coexpressing AD/AD-FHY3/AD-FAR1 and AD/HY5/AD-HY5 protein combinations shown on the left. Error bars represent SD (n = 4).

(C) Structure of the dual-luciferase reporter construct in which the firefly luciferase (*LUC*) reporter gene is driven by the wild type or ACE-mutated (both of the ACGT elements were mutated to AaaT) *FHY1-B* promoter fragment. The *Renillia* luciferase (*REN*) reporter gene is controlled by the constitutive 35S promoter. A 105-bp (−101 to +4) *NOS* minimal promoter (Puente et al., 1996) was inserted upstream of the *LUC* coding sequence to allow the promoter fragment to drive the *LUC* reporter gene transcription. Cauliflower mosaic virus terminator (Ter) and the T-DNA left border (LB) and right border (RB) are also indicated.

(D) Relative reporter activity in tobacco cells transiently transformed with the indicated effector and reporter constructs. FHY3 and HY5 are expressed by the *35S:FHY3* and *35S:HY5* effector plasmids (see Methods), respectively. Tobacco leaves were kept in white light for 4 d after infiltration. The relative LUC activities normalized to the REN activity are shown (LUC/REN). Error bars represent SD (n = 3).

[See online article for color version of this figure.]

AD-FAR1 robustly activate *LacZ* reporter gene expression in yeast cells. However, coexpression of HY5 with AD-FHY3 or AD-FAR1 significantly decreases β-galactosidase activity (at least $P < 0.05$ for each compared group), implying that HY5 negatively regulates FHY3/FAR1-activated *FHY1*/*FHL* expression. Moreover, if an activation domain was added to HY5, the reporter gene expression was increased (Figures 5A and 5B), indicating that HY5 does occupy the *FHY1*/*FHL* promoters when it is coexpressed with FHY3/FAR1 in yeast cells.

We conducted a transient transcription assay in *Nicotiana benthamiana* leaves to study whether HY5 plays a similar regulatory role in plant cells. We generated dual-luciferase reporter constructs to allow the wild-type or ACE-mutated B fragment of the *FHY1* promoter to drive *LUC* reporter gene expression (Figure 5C). As shown in Figure 5D, transiently expressed FHY3 acts as an activator of the *FHY1* promoter. HY5 alone, however, does not show an obvious effect on transcription, consistent with its behavior in yeast cells. Coexpression of HY5 with FHY3 dramatically decreases the reporter gene expression to a similar level as HY5 alone, and this contrast is especially obvious when the ACEs in the *FHY1p-B* promoter fragment are mutated (Figure 5D).

Together with the EMSA assay data shown in Figure 4, we conclude that HY5 negatively regulates *FHY1*/*FHL* expression via two mechanisms. First, HY5 binds to the *FHY1*/*FHL* promoters, and as the binding sites of HY5 are in close proximity to those of FHY3/FAR1 in the *FHY*/*FHL* promoters, HY5's occupation consequently decreases the accessibility of the promoters to FHY3/FAR1. Second, HY5's interaction with FHY3/FAR1 may prevent them from binding to the *FHY*/*FHL* promoters. These two mechanisms are quite distinct from each other, but both seem important as HY5 could downregulate *FHY1*/*FHL* expression with or without its binding sites in the *FHY1*/*FHL* promoters provided FHY3/FAR1 are present (Figure 5). In either case, HY5 achieves its regulatory goal by modulating the activities of the transcriptional activators FHY3 and FAR1.

HY5 Negatively Regulates *FHY1*/*FHL* Transcript and FHY1 Protein Levels in Vivo

To confirm the role of HY5 in the regulation of *FHY1*/*FHL* expression in vivo, we compared the *FHY1* and *FHL* transcript levels in wild-type and *hy5* mutant seedlings by RNA gel blot and qRT-PCR analyses. Our data show that *FHY1* and *FHL* transcript

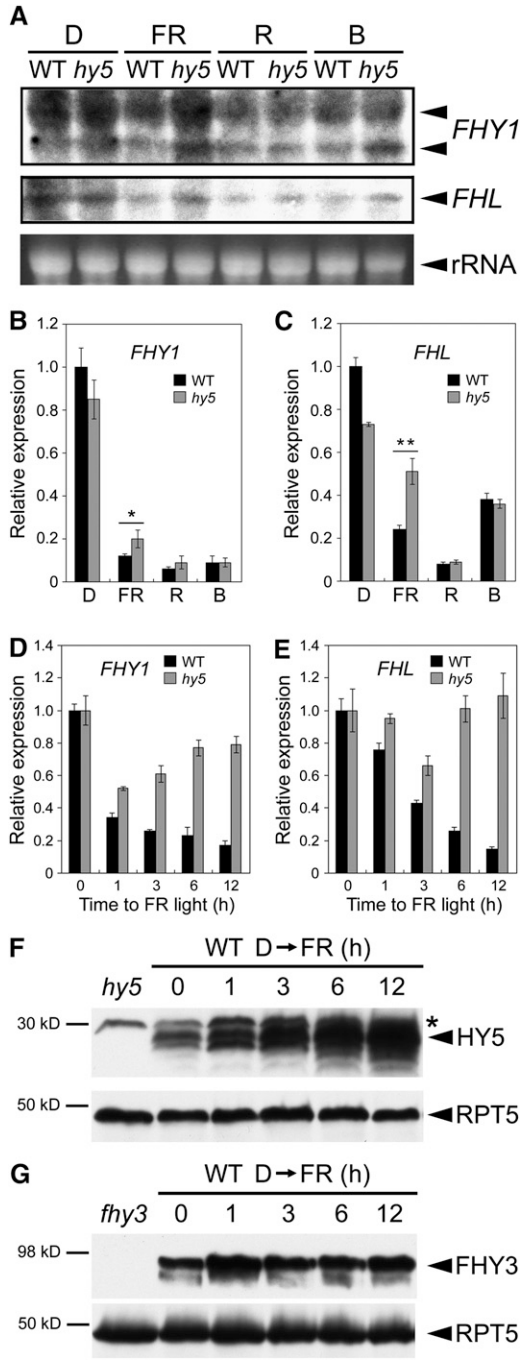

Figure 6. HY5 Negatively Regulates *FHY1* and *FHL* Transcript Levels in Vivo.

(A) RNA gel blot analysis showing *FHY1* and *FHL* mRNA levels in 4-d-old wild-type (WT) and *hy5* mutant seedlings grown in darkness (D) or continuous FR, R, and B light conditions. Ethidium bromide staining showing rRNA was used as the loading control.

(B) and **(C)** Real-time qRT-PCR analysis showing *FHY1* and *FHL* transcript levels in wild-type and *hy5* mutant seedlings. Error bars represent SD of triplicate experiments. *P < 0.05 and **P < 0.01 (Student's *t* test) for the indicated pair of seedlings.

(D) and **(E)** Changes in *FHY1* and *FHL* transcript levels in wild-type and *hy5* mutant seedlings grown in darkness for 4 d and then transferred to FR light for various time periods. The expression levels in dark-grown seedlings were set as 1. Error bars represent SD of triplicate experiments.

(F) and **(G)** Immunoblots showing the changes of HY5 **(F)** and FHY3 **(G)** protein levels in wild-type seedlings grown in darkness for 4 d and then subjected to FR light treatment for various time periods. The mutant plants (*hy5-215* and *fhy3-1*, respectively) were included as negative controls for immunoblots. Asterisk in **(F)** indicates a band cross-reacting with HY5 antibody. Anti-RPT5 was used as a sample loading control.

levels are notably elevated in *hy5* mutant seedlings particularly under continuous FR light (Figures 6A to 6C), indicating that HY5 indeed negatively regulates *FHY1* and *FHL* expression in *Arabidopsis*.

Previous reports showed that *FHY1* and *FHL* transcript levels declined rapidly when the dark-grown wild-type seedlings were exposed to FR light (Desnos et al., 2001; Lin et al., 2007). We confirmed this pattern of *FHY1*/*FHL* downregulation using qRT-PCR (Figures 6D and 6E). To investigate the role of HY5 in this process, we examined the expression of *FHY1* and *FHL* in *hy5* mutant seedlings subjected to the same dark-to-FR light treatment. Intriguingly, expression of both *FHY1* and *FHL* in *hy5* mutants showed a lesser decrease compared with wild-type plants during the first 1 and 3 h, respectively, and then obviously increased during subsequent FR light treatment (Figures 6D and 6E), indicating that HY5 does play a major role in downregulating *FHY1*/*FHL* transcript levels in this FR light exposure treatment.

We then examined the levels of HY5 and FHY3 proteins in this time course to rule out the possibility that HY5 might downregulate *FHY1*/*FHL* transcript levels indirectly by downregulating *FHY3* and *FAR1* expression, as the expression of *FHY3* and *FAR1* displayed a pattern similar to that of *FHY1* and *FHL* in this process (Lin et al., 2007). Our immunoblot data show that HY5 protein levels increased dramatically and continuously after the dark-grown wild-type seedlings were transferred to FR light (Figure 6F). By contrast, FHY3 protein levels showed a mild increase after 1 h of FR light exposure and then remained relatively stable in the subsequent FR light treatment, at least without showing an obvious decrease (Figure 6G). These data suggest that HY5 directly exerts its regulation on the *FHY1* and *FHL* promoters, rather than indirectly through regulating expression of *FHY3* and *FAR1*, although we cannot rule out the possibility that HY5 may regulate *FHY3* and *FAR1* transcript levels as well.

We further examined whether the abundance of FHY1 protein is correspondingly regulated by HY5. As reported in a recent study from our group (Shen et al., 2009), our FHY1 antibodies always recognize two endogenous FHY1 bands in immunoblots. Interestingly, only the smaller FHY1 band seems to be regulated by the *hy5* mutation in different light conditions (Figure 7). Moreover, the abundance of the smaller FHY1 band was increased in *hy5* mutants not only in continuous FR light but also in R and B light conditions, suggesting that HY5 may be involved in the posttranscriptional regulation of FHY1 as well.

We also tested FHY1 protein accumulation in *fhy3 far1* and *phyA* mutants. Consistent with the previous reports that FHY3 and FAR1 are key positive regulators of *FHY1* expression

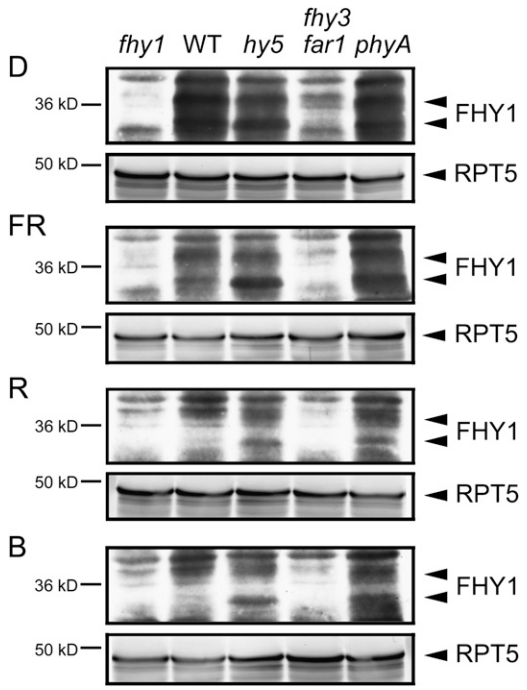

Figure 7. HY5 Negatively Regulates FHY1 Protein Levels.

FHY1 protein levels in 4-d-old *fhy1*, wild-type (WT), *hy5*, *fhy3 far1*, and *phyA* seedlings grown in darkness (D) or continuous FR, R, and B light conditions. The two arrowheads indicate two endogenous FHY1 bands in immunoblots recognized by our FHY1 antibodies (Shen et al., 2009). Anti-RPT5 was used as a sample loading control.

(Desnos et al., 2001; Zhou et al., 2005; Lin et al., 2007), FHY1 protein level is severely attenuated in *fhy3 far1* double mutants in darkness and all light conditions, comparable to that in *fhy1* mutants (Figure 7). However, phyA only downregulates FHY1 protein level in light conditions (Figure 7), which might be achieved by direct phosphorylation of FHY1 by phyA under light conditions (Shen et al., 2009).

COP1 Positively Regulates *FHY1*/*FHL* Transcript Levels in Darkness

As HY5 was targeted for degradation by COP1 in darkness (Osterlund et al., 2000), and HY5 acts as a repressor of *FHY1*/*FHL* expression, we next examined whether COP1 positively regulates *FHY1*/*FHL* expression in darkness indirectly via HY5. To this end, we first confirmed that, as reported previously, HY5 protein level was extraordinarily elevated in *cop1* mutants in darkness (Osterlund et al., 2000; see Supplemental Figure 3 online), while FHY3 protein level was not decreased by the *cop1* mutation (data not shown). Then, we examined *FHY1* and *FHL* transcript levels in dark-grown wild-type and *cop1* mutant seedlings. Intriguingly, both *FHY1* and *FHL* transcript levels in *cop1* mutants were decreased to around 10% of that in the wild type (Figures 8A and 8B), suggesting that HY5 may play a major role in mediating COP1-regulated *FHY1*/*FHL* expression. Consistent with this finding, our previous study shows that COP1 is essential for FHY1 protein accumulation in darkness (Shen et al., 2005).

As the other COP/DET/FUS proteins, such as DET1, COP10, and the subunits of the COP9 signalosome, were shown to be required for degradation of HY5 in darkness (Osterlund et al., 2000), it is likely that these COP/DET/FUS proteins may positively regulate *FHY1*/*FHL* expression in darkness as well. Consistent with this assumption, our previous study also showed that these COP/DET/FUS proteins are required for normal accumulation of FHY1 protein in darkness (Shen et al., 2005).

HY5 Repression of *FHY1* Expression Requires the Presence of FHY3 and FAR1

As discussed above, since HY5 lacks transcriptional repression activity in yeast and plant cells (Figure 5), it seems that HY5 represses *FHY1*/*FHL* expression by modulating the activities of their transcriptional activators FHY3 and FAR1. To test this hypothesis in vivo, we generated *hy5 fhy3 far1* triple mutants to mutate both the positive regulators FHY3/FAR1 and the negative regulator HY5 of *FHY1*/*FHL* expression (see Supplemental Figure 4 online). We selected two independent triple mutant lines and confirmed that all three loci are homozygous in both lines (Figure 9A). qRT-PCR data show that in *hy5 fhy3 far1* triple mutants, *FHY1* transcript levels are similar to those in *fhy3 far1* double mutants (Figure 9B), suggesting that HY5 repression of *FHY1* expression requires the presence of its transcriptional activators FHY3 and FAR1 in vivo.

DISCUSSION

FHY1 and FHL are two small proteins (202 and 201 amino acids, respectively) in *Arabidopsis* that were found to have homologs in both monocot and dicot plant species (Genoud et al., 2008). It

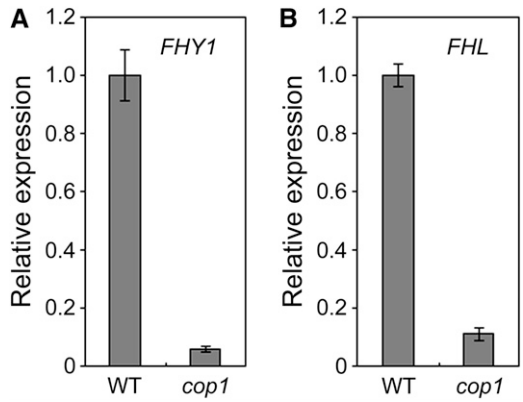

Figure 8. COP1 Positively Regulates *FHY1* and *FHL* Transcript Levels in Darkness.

Real-time qRT-PCR analysis showing that *FHY1* **(A)** and *FHL* **(B)** transcript levels were markedly decreased in dark-grown *cop1* mutants compared with wild-type (WT) plants. Error bars represent SD of triplicate experiments.

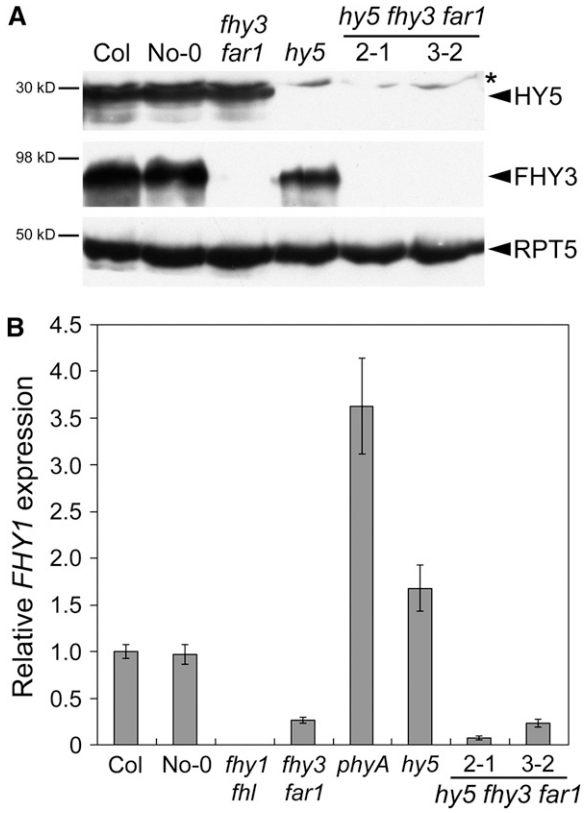

Figure 9. HY5 Repression of *FHY1* Expression Requires the Presence of FHY3 and FAR1.

(A) Detection of HY5 and FHY3 proteins in 4-d-old wild-type (Col and No-0 ecotypes), *fhy3 far1*, *hy5*, and two independent homozygous lines of *hy5 fhy3 far1* triple mutant plants grown under continuous FR light. Anti-RPT5 was used as a sample loading control. The *far1-2* mutation in the two lines of *hy5 fhy3 far1* triple mutants was confirmed by directly sequencing the PCR products that contain the mutation (data not shown).
(B) Real-time qRT-PCR analysis showing *FHY1* transcript levels in 4-d-old wild-type (Col and No-0 ecotypes), *fhy1 fhl*, *fhy3 far1*, *phyA*, *hy5*, and two independent homozygous lines of *hy5 fhy3 far1* triple mutant plants grown under continuous FR light. Error bars represent SD of triplicate experiments.

was shown that FHY1 and FHL are required for nuclear accumulation of phyA since phyA is localized only in the cytosol of *fhy1 fhl* double mutants (Hiltbrunner et al., 2006; Rösler et al., 2007). This finding was greatly extended by a recent report that FHY3 and FAR1, two transposase-derived transcription factors, are the key activators of *FHY1*/*FHL* transcription and thus indirectly regulate phyA nuclear accumulation and phyA signaling (Lin et al., 2007). However, the mechanism by which *FHY1*/*FHL* expression is downregulated by the feedback regulation of phyA signaling remains unclear, although previous reports indicate that expression of *FHY1*/*FHL*, as well as *FHY3*/*FAR1*, are under this control (Desnos et al., 2001; Lin et al., 2007).

In this study, we show that the well-characterized *Arabidopsis* bZIP transcription factor HY5 directly represses *FHY1*/*FHL* expression in FR light, and, interestingly, this action of HY5 is accomplished by modulating the activities of the transcriptional activators FHY3 and FAR1. Because FHY1 and FHL are the key positive regulators of phyA signaling, the consequence of this action of HY5 may potentially attenuate phyA signaling. However, HY5 has been genetically defined as a positive regulator of phyA signaling, as mutations in *HY5* cause a defect in the inhibition of hypocotyl elongation in continuous FR light (Oyama et al., 1997; Ang et al., 1998; see Supplemental Figure 4 online). In fact, our results may not be contradictory to the previous findings because this role of HY5 in downregulating *FHY1*/*FHL* transcript levels serves mainly in the feedback process of phyA signaling (i.e., phyA signaling has already been triggered and, thus, *FHY1*/*FHL* transcripts are not required at high levels), as it was shown that the accumulation of HY5 protein in FR light also requires phyA (Osterlund et al., 2000). Thus, once phyA is imported into the nucleus by FHY1 and FHL, phyA triggers a signaling cascade, and one consequence of this cascade is the accumulation of HY5, which acts to promote photomorphogenesis and downregulate *FHY1*/*FHL* transcript levels simultaneously (Figure 10). Therefore, our data suggest that HY5 plays dual roles in phyA signaling.

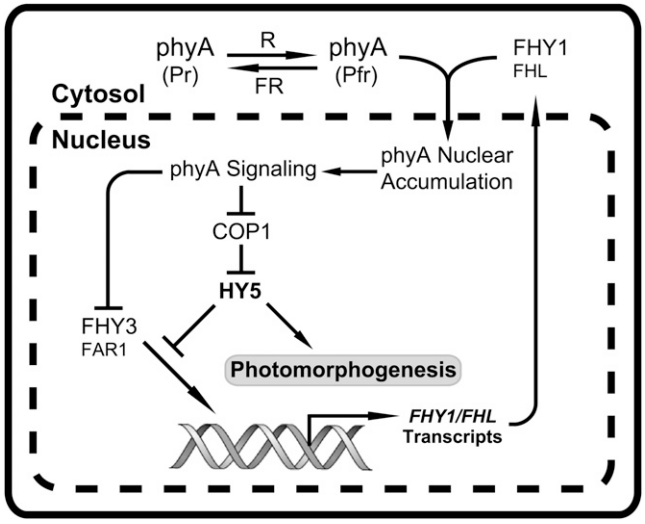

Figure 10. A Working Model Depicting How HY5 Functions in the Feedback Regulation of phyA Signaling.

In the absence of light, FHY3 and FAR1 induce the expression of *FHY1* and *FHL* in anticipation of the upcoming light signal. Accumulation of FHY1 and FHL proteins in dark-grown seedlings may serve to ensure rapid and sufficient phyA nuclear accumulation upon FR light exposure to jump start phyA signaling events in the nucleus. Upon light exposure, the Pfr form of phyA is imported into the nucleus by FHY1/FHL and thus triggers phyA signaling leading to multiple light responses, including the reduction of COP1 in the nucleus and accumulation of HY5 (Osterlund and Deng, 1998; Osterlund et al., 2000), and feedback regulation of *FHY3* and *FAR1* transcript levels (Lin et al., 2007). HY5 plays dual roles in phyA signaling: promoting photomorphogenesis and downregulating *FHY1*/*FHL* transcript levels by modulating the activities of the transcriptional activators FHY3 and FAR1. FHY3 and FHY1 (indicated by larger letters) are the more predominant players in the phyA signaling process compared with their respective homologs FAR1 and FHL. Pr, R-absorbing form of phyA (inactive); Pfr, FR-absorbing form of phyA (active). Arrow, positive regulation; bar, negative regulation.

Because FHY1 and FHL regulate phyA nuclear accumulation, and HY5 negatively regulates FHY1 (and possibly FHL) protein levels (Figure 7), it is reasonable to propose that HY5 might negatively regulate phyA nuclear accumulation. However, we failed to detect any difference in nuclear phyA levels between FR-grown wild-type and *hy5* mutant seedlings in our nuclear fractionation assays (data not shown). This result may be explained by two possibilities. First, nuclear phyA levels may be tightly controlled, as one molecular event after phyA is imported into the nucleus by FHY1/FHL is the degradation of the photoreceptor (Seo et al., 2004; Saijo et al., 2008; Debrieux and Fankhauser, 2010). Thus, the excess phyA proteins imported into the nucleus by the increased levels of FHY1/FHL proteins may be degraded rapidly. Second, overaccumulation of FHY1/FHL may affect mainly the kinetics of phyA nuclear accumulation, rather than the steady state levels of nuclear phyA, whereas nuclear fractionation assays only roughly detect the steady state levels of nuclear phyA. So the question whether HY5 regulates phyA nuclear accumulation will require further investigation and better techniques.

We show that HY5 acts as a repressor of *FHY1*/*FHL* expression. However, HY5 itself does not show any transcriptional activity in yeast and plant cells (Figure 5). Our data suggest that HY5 achieves its regulatory effects by modulating the activities of FHY3/FAR1, two activators of *FHY1*/*FHL* expression. This conclusion is also supported by the examination of *FHY1* expression in *hy5 fhy3 far1* triple mutants (Figure 9). In another report, HY5 was shown to be necessary for normal circadian expression of the *Lhcb* genes via interaction with CCA1 (Andronis et al., 2008). Consistent with these findings, a recent study demonstrates that HY5 binding to the target gene promoters is not sufficient for transcriptional regulation, implying that HY5 may need other cofactors to regulate target gene expression (Lee et al., 2007). Moreover, a transcription factor protein microarray study discovered 20 transcription factor candidates that may interact with HY5 (Gong et al., 2008). Thus, the discovery of more HY5-interacting cofactors and elucidation of more regulatory modes of HY5 may help in understanding how HY5 implements its hierarchical role in promoting photomorphogenesis.

In summary, our work reveals an interesting new role of HY5 in the feedback regulation of phyA signaling. Taken together with the previous reports, the feedback regulation of phyA signaling is achieved by at least four distinct mechanisms: negative regulation of *FHY1*/*FHL* expression by HY5 shown in this study, *FHY3*/*FAR1* repression by phyA signaling (Lin et al., 2007), direct phosphorylation of FHY1 by phyA and subsequent FHY1 degradation (Shen et al., 2005, 2009), and phosphorylation and subsequent degradation of phyA itself (Saijo et al., 2008). The multiple layers of feedback regulation imply a complicated and delicate fine-tuned control of phyA signaling, thus allowing plants to respond quickly, appropriately, and precisely to their dynamic light environment.

METHODS

Plant Materials and Growth Conditions

The wild-type *Arabidopsis thaliana* used in this study is of the Columbia (Col) ecotype, unless otherwise indicated. The *phyA-211* (Reed et al., 1994), *fhy1-3* (Zeidler et al., 2001), *fhy1-3 fhl-1* (Rösler et al., 2007), *hy5-215* (Oyama et al., 1997), *fhy3-1* (Whitelam et al., 1993), and *cop1-4* (McNellis et al., 1994) mutants are of the Col ecotype, and the *fhy3-4* (Wang and Deng, 2002) and *fhy3-4 far1-2* (Lin et al., 2007) mutants are of the No-0 ecotype and have been described previously. The *hy5 fhy3 far1* triple mutant was constructed by crossing *hy5-215* and *fhy3-4 far1-2* mutants. The mutation *hy5-ks50* (Oyama et al., 1997) was introduced into the Col background by genetic backcrossing (Lee et al., 2007). The growth conditions and light sources were as described previously (Shen et al., 2005).

Plasmid Construction

The *FHY1p:LacZ* and *FHLp:LacZ* reporter constructs were described previously (Lin et al., 2007). To generate *FHY1p-A:LacZ*, *FHY1p-B:LacZ*, *FHY1p-C:LacZ*, *FHY1p-D:LacZ*, *FHLp-A:LacZ*, *FHLp-B:LacZ*, *FHLp-C:LacZ*, and *FHLp-D:LacZ* reporter constructs, the promoter fragments were amplified by PCR using *FHY1p:LacZ* and *FHLp:LacZ* constructs as the templates and the respective pairs of primers (see Supplemental Table 1 online), and then cloned into the *Eco*RI-*Xho*I sites of the pLacZi2μ vector (Lin et al., 2007), respectively. To generate various *LacZ* reporter genes driven by the wild-type and mutant subfragments of the *FHY1* and *FHL* promoters shown in Figure 2B, oligonucleotides were synthesized as two complementary oligo primers with an *Eco*RI site overhang at the 5′ end and an *Xho*I site overhang at the 3′ end (see Supplemental Table 1 online). The oligo primers were annealed, and the double-stranded oligonucleotides were ligated into the *Eco*RI-*Xho*I sites of the pLacZi2μ vector, producing *FHY1p-ACE1WT:LacZ*, *FHY1p-ACE2WT:LacZ*, *FHLp-ACE1WT:LacZ*, *FHLp-ACE2WT:LacZ*, *FHLp-ACE3WT:LacZ*, *FHY1p-ACE1mut:LacZ*, *FHY1p-ACE2mut:LacZ*, *FHLp-ACE1mut:LacZ*, *FHLp-ACE2mut:LacZ*, and *FHLp-ACE3mut:LacZ*, respectively.

To mutate the respective ACEs in the B fragment of the *FHY1* promoter, the *FHY1p-B:LacZ* reporter plasmid (described above) was used as the template using the QuikChange site-directed mutagenesis kit (Stratagene) and the primers shown in Supplemental Table 1 online according to the manufacturer's instructions. To generate the *FHY1p-B(ACEm):LacZ* reporter construct in which both of the ACGT elements were mutated into AaaT, two rounds of mutagenesis reactions were performed, with each round introducing one mutation into the promoter.

The AD-FHY3, AD-FAR1, and AD-COP1 constructs were described previously (Ang et al., 1998; Wang and Deng, 2002). To generate AD-PIF3, AD-HY5, AD-LAF1, AD-HFR1, and AD-HYH, the full-length coding sequences of PIF3, HY5, LAF1, HFR1, and HYH were amplified by PCR with the respective pairs of primers (see Supplemental Table 1 online) and then cloned into the *Eco*RI-*Xho*I sites of the pB42AD vector (Clontech), respectively. To generate pGAD-T7 constructs for expressing HY5 and AD-HY5 fusion proteins in yeast cells, the full-length coding sequence of HY5 was amplified by PCR with the respective pairs of primers (see Supplemental Table 1 online) and then cloned into the *Kpn*I-*Xho*I and *Eco*RI-*Xho*I sites of the pGAD-T7 vector (Clontech), respectively.

The LexA-HY5, LexA-HY5N, and LexA-HY5C constructs were described previously (Ang et al., 1998). To generate LexA-FHY3N, LexA-FHY3C1, and LexA-FHY3C2, the fragments were amplified by PCR with the respective pairs of primers (see Supplemental Table 1 online) and then cloned into the *Bam*HI-*Sal*I sites of the pLexA vector (Clontech), respectively. To generate LexA-FAR1N, LexA-FAR1C1, and LexA-FAR1C2, the respective PCR fragments were cloned into the *Bam*HI-*Xho*I sites of the pLexA vector, respectively.

The GST-HY5 and GST-FHY3N constructs were described previously (Ang et al., 1998; Lin et al., 2007). To generate GST-HY5C, the PCR fragment was cloned into the *Bam*HI-*Xho*I sites of the pGEX-4T-1 vector (Amersham Biosciences). To generate 6×His-FHY3N and 6×His-FAR1N, the PCR fragments were cloned into the *Bam*HI-*Sal*I and *Bam*HI-*Xho*I sites of the pET-28a vector (Novagen), respectively.

To generate HY5-NLuc, a *Bam*HI-*Sal*I PCR fragment of full-length HY5 was cloned into the corresponding sites of the vector 35S:NLuc (Chen et al., 2008). To generate CLuc-FHY3N, a *Kpn*I-*Sal*I PCR fragment of FHY3N (first 260 amino acids) was cloned into the corresponding sites of the vector 35S:CLuc (Chen et al., 2008).

To generate the *35S:FHY3* vector, a *Bam*HI-*Sal*I fragment containing the full-length coding sequence of FHY3 was released from LexA-FHY3 (Wang and Deng, 2002), and then the released fragment was inserted into the *Bam*HI-*Sal*I sites of the pSPYNE-35S vector (Walter et al., 2004). To generate the *35S:HY5* vector, a *Bam*HI-*Sal*I PCR fragment of full-length HY5 was cloned into the corresponding sites of the pSPYNE-35S vector.

To generate the *FHY1p-B:LUC* and *FHY1p-B(ACEm):LUC* reporter constructs, a *Pst*I-*Bam*HI PCR fragment containing the 105 bp (−101 to +4) *NOS* minimal promoter was amplified from *NOS101-GUS* (Puente et al., 1996) and inserted into the corresponding sites of pGreenII 0800-LUC (Hellens et al., 2005), resulting in miniPro:LUC vector. Then, the B fragment of the *FHY1* promoter was amplified by PCR using *FHY1p-B:LacZ* and *FHY1p-B(ACEm):LacZ* constructs (described above) as the templates and cloned into the *Kpn*I-*Xho*I sites of miniPro:LUC to produce the *FHY1p-B:LUC* and *FHY1p-B(ACEm):LUC* vectors, respectively.

All of the primers used to generate the above-mentioned constructs are listed in Supplemental Table 1 online, and all of the constructs were confirmed by sequencing prior to usage in various assays.

Yeast Assays

For yeast one-hybrid assays, plasmids for AD fusions were cotransformed with the *LacZ* reporter genes driven by various *FHY1* and *FHL* promoter fragments into the yeast strain EGY48; for yeast two-hybrid assays, the respective combinations of LexA and AD fusion plasmids were cotransformed into the yeast strain EGY48, which already contains the reporter plasmid *p8op:LacZ* (Clontech). Transformants were grown on proper dropout plates containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for blue color development. Yeast transformation and liquid assay were conducted as described in the Yeast Protocols Handbook (Clontech).

Immunoblotting

For anti-FHY3 immunoblots, *Arabidopsis* seedlings were ground to a fine powder and total proteins were eluted in 2× SDS loading buffer. For all the other immunoblots, *Arabidopsis* seedlings were homogenized in an extraction buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 0.1% Tween 20, 1 mM PMSF, 40 μM MG132, and 1× complete protease inhibitor cocktail (Roche). Immunoblotting was performed as previously described (Shen et al., 2005).

Primary antibodies used in this study include anti-FHY1 (Shen et al., 2005), anti-HY5 (Osterlund et al., 2000), anti-FHY3 (Saijo et al., 2008), and anti-RPT5 (Kwok et al., 1999) antibodies.

ChIP

Wild-type seedlings grown under continuous FR, R, and B light conditions for 4 d were used for ChIP assays following the procedure described previously (Lee et al., 2007). Briefly, 2 g of seedlings grown under the indicated light conditions were first cross-linked with 1% formaldehyde under vacuum. The samples were ground to powder in liquid nitrogen, and the chromatin complexes were isolated and sonicated and then incubated with polyclonal HY5 antibodies (Osterlund et al., 2000). The precipitated DNA was recovered and analyzed by PCR methods using the primers in Supplemental Table 1 online. Real-time qPCR analysis was performed using the respective pair of primers and Power SYBR Green PCR Master Mix (Applied Biosystems) with a Bio-Rad CFX96 real-time PCR detection system. PCR reactions were performed in triplicate for each sample, and the ChIP values were normalized to their respective DNA input values.

Preparation of Recombinant Proteins

All constructs were transformed into *Escherichia coli* BL21 (DE3) cells that were treated with isopropyl-β-D-thiogalactoside to induce fusion protein expression. The GST fusion proteins were purified with Glutathione Sepharose 4B beads (Amersham Biosciences), and the 6×His-fusion proteins were purified with nickel-nitrilotriacetic acid beads (Qiagen).

EMSA

EMSAs were performed using biotin-labeled probes and the Lightshift Chemiluminescent EMSA kit (Pierce). The sequences of the complementary oligonucleotides used to generate the biotin-labeled and unlabeled probes are shown in Supplemental Table 1 online. Briefly, 0.5 μg of GST or GST fusion proteins were incubated together with biotin-labeled probes in 20-μL reaction mixtures containing 10 mM Tris-HCl, 150 mM KCl, 1 mM DTT, 50 ng/μL poly (dI-dC), 2.5% glycerol, 0.05% Nonidet P-40, 100 μM $ZnCl_2$, and 0.5 μg/μL BSA for 20 min at room temperature and separated on 6% native polyacrylamide gels in Tris-glycine buffer. For the competition assays shown in Figure 4B, 0.5, 1, and 2 μg of GST or GST-HY5C proteins were used, respectively. The labeled probes were detected according to the instructions provided with the EMSA kit.

RNA Gel Blot Analysis and Real-Time qRT-PCR

Total RNA was extracted from *Arabidopsis* seedlings using the RNeasy plant mini kit (Qiagen). For RNA gel blot analysis, 15 μg of total RNA were loaded per lane and blotting was performed as described previously (Martínez et al., 2004). Fragments of *FHY1* and *FHL* used for probe labeling were generated by PCR, and the primers are shown in Supplemental Table 1 online.

For real-time qRT-PCR, cDNAs were synthesized from 2 μg total RNA using SuperScript II first-strand cDNA synthesis system (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed as described above. PCR reactions were performed in triplicate for each sample, and the expression levels were normalized to that of a *ubiquitin* gene.

In Vitro Pull-Down Assay

For in vitro binding, 2 μg of purified recombinant bait proteins (GST-HY5 and GST) and 2 μg of prey proteins (6×His-FHY3N and 6×His-FAR1N) were added to 1 mL of binding buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.6% Triton X-100. After incubation at 4°C for 2 h, Glutathione Sepharose 4B beads (Amersham Biosciences) were then added and incubated for a further 1 h. After washing three times with the binding buffer, pulled-down proteins were eluted in 2× SDS loading buffer at 95°C for 10 min, separated on 10% SDS-PAGE gels, and detected by immunoblotting using anti-His antibody (Qiagen).

LCI Assay

Transient LCI assays in *Nicotiana benthamiana* were performed as described previously (Chen et al., 2008). Briefly, *Agrobacterium tumefaciens* (strain GV2260) bacteria containing indicated constructs were infiltrated into young but fully expanded leaves of the 7-week-old *N. benthamiana* plants using a needleless syringe. After infiltration, plants were grown under 16-h light/dark for 3 d, and luciferase signals were then

viewed in an IVIS Spectrum imaging system (Caliper LifeSciences) and quantified with the Living Image 4.0 software. To confirm the expression of the NLuc and CLuc fusion proteins, total protein was extracted from equal amounts of tobacco leaves and subjected to immunoblot analysis with anti- firefly LUC antibodies (Sigma-Aldrich), which react with both the N- and C-terminal firefly LUC fragments. The amount of protein loaded in each lane is indicated by Ponceau S staining of ribulose-1,5-bisphosphate carboxylase/oxygenase.

Transient Transcription Dual-Luciferase Assay

Transient dual-luciferase assay in *N. benthamiana* was performed as described previously (Hellens et al., 2005). After infiltration, plants were left under continuous white light for 4 d, and then leaf samples were collected. Firefly luciferase and *Renillia* luciferase were assayed using the dual luciferase assay reagents (Promega) and were performed essentially as previously described (Liu et al., 2008). Briefly, leaf discs (1 to 2 cm in diameter) were excised, ground in liquid nitrogen, and homogenized in 100 μL of the Passive Lysis Buffer. Eight microliters of this crude extract was mixed with 40 μL of Luciferase Assay Buffer, and the firefly luciferase (LUC) activity was measured using a GLOMAX 20/20 luminometer (Promega). Forty microliters of Stop and Glow Buffer was then added to the reaction, and the *Renillia* luciferase (REN) activity was measured. Three biological repeats were measured for each sample.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: FHY1 (At2g37678), FHL (At5g02200), FHY3 (At3g22170), FAR1 (At4g15090), HY5 (At5g11260), PIF3 (At1g09530), LAF1 (At4g25560), HFR1 (At1g02340), COP1 (At2g32950), and PHYA (At1g09570).

Supplemental Data

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

Supplemental Figure 1. HYH Does Not Bind to the *FHY1* Promoter.

Supplemental Figure 2. HY5 Protein Levels in Wild-Type Seedlings Grown in Different Light Conditions.

Supplemental Figure 3. HY5 Protein Levels in Wild-Type and *cop1* Mutant Seedlings in Darkness.

Supplemental Figure 4. Phenotypes of the Various *Arabidopsis* Mutants Defective in phyA Signaling.

Supplemental Table 1. Summary of Primers Used in This Study.

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