Transposase-Derived Proteins FHY3/FAR1 Interact with PHYTOCHROME-INTERACTING FACTOR1 to Regulate Chlorophyll Biosynthesis by Modulating HEMB1 during Deetiolation in Arabidopsis*

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Successful chlorophyll biosynthesis during initial light exposure is critical for plant survival and growth, as excess accumulation of chlorophyll precursors in darkness can cause photooxidative damage to cells. Therefore, efficient mechanisms have evolved to precisely regulate chlorophyll biosynthesis in plants. Here, we identify FAR-RED ELONGATED HYPOCOTYL3 (FHY3) and FAR-RED IMPAIRED RESPONSE1 (FAR1), two transposase-derived transcription factors, as positive regulators of chlorophyll biosynthesis in Arabidopsis thaliana. We show that null mutations in FHY3 and FAR1 cause reduced protochlorophyllide (a precursor of chlorophyll) levels in darkness and less photobleaching in the light. We find that FHY3 directly binds to the promoter and activates expression of HEMB1, which encodes 5-aminolevulinic acid dehydratase in the chlorophyll biosynthetic pathway. We reveal that PHYTOCHROME-INTERACTING FACTOR1 physically interacts with the DNA binding domain of FHY3, thereby partly repressing FHY3/FAR1-activated HEMB1 expression. Strikingly, FHY3 expression is upregulated by white light. In addition, our genetic data indicate that overexpression, severe reduction, or lack of HEMB1 impairs plant growth and development. Together, our findings reveal a crucial role of FHY3/FAR1 in regulating chlorophyll biosynthesis, thus uncovering a new layer of regulation by which light promotes plant dark-light transition in early seedling development.

INTRODUCTION

As sessile organisms, plants respond to the surrounding environments by shaping their growth and development. Light is one of the major environmental signals that influences plants throughout their life cycle, from seed germination to flowering. Under the soil, germinating seedlings undergo deetiolation (also called skotomorphogenesis) with long hypocotyls and closed cotyledons lacking chlorophyll and functional chloroplasts. Upon emerging from the soil and reaching light, the etiolated seedlings undergo deetiolation (also termed photomorphogenesis), including cotyledon opening, chlorophyll biosynthesis, the development of chloroplasts, and subsequently autotrophic growth (Von Arnim and Deng, 1996, Casal et al., 2004).

Phytochrome and cryptochrome photoreceptors are responsible for perceiving and transducing light signals to regulate distinct photomorphogenic responses. In Arabidopsis thaliana, phytochrome A (phyA) to phyE form a small protein family that predominantly regulates various responses to red and far-red light, whereas the cryptochromes (cry1 and cry2) absorb the blue/UV-A light (Whitelam et al., 1993; Neff et al., 2000; Lin 2002). phyA is the primary photoreceptor for mediating far-red light signaling. FAR-RED ELONGATED HYPOCOTYL3 (FHY3) and FAR-RED-IMPAIRED RESPONSE1 (FAR1) are homologous proteins that function as positive regulators and act early in phyA signaling (Hudson et al., 1998; Wang and Deng, 2002; Wang et al., 2002). They work together to modulate phyA nuclear accumulation and phyA responses through directly activating gene expression of a pair of downstream targets, FYH1 and FYH1-LIKE (Lin et al., 2007, 2008). Studies also showed that FHY3 and FAR1 integrate light signals into the circadian clock and modulate chloroplast division by directly upregulating expression of EARLY FLOWERING4 and ACCUMULATION AND REPLICATION OF CHLOROPLASTS (ARCS), respectively (Allen et al., 2006; Li et al., 2011; Ouyang, et al., 2011). We and others previously documented that FHY3 and FAR1 define a type of transcription factors that were derived from ancient transposases during evolution and may play diverse roles (Hudson et al., 2003; Lin et al., 2007). A recent study found that FHY3 has more than a thousand putative direct targets in Arabidopsis (Ouyang et al., 2011), implicating it as having broad functions in plant growth and development, most of which, however, are unknown.
Chlorophyll formation is a hallmark of the photomorphogenic response, and chlorophylls serve as the major pigments in photosynthesis by harvesting light energy and driving electron transfer. Chlorophyll metabolism has been extensively studied with various organisms biochemically and genetically (Eckhardt et al., 2004; Tanaka and Tanaka, 2006, 2007). Chlorophyll biosynthesis shares early steps from the first committed precursor 5-aminolevulinic acid (ALA) to protoporphyrin IX with that of heme, siroheme, and phytochromobilin in the tetrapyrrole biosynthetic pathway (Battersby, 2000; Tanaka and Tanaka, 2007; Mochizuki et al., 2010). Two molecules of ALA are then condensed to form a pyrrole molecule, porphobilinogen (PBG), by ALA dehydratase (ALAD). After sequential enzymatic conversions, the pathway is divided by metal chelation reactions of protoporphyrin IX, thereby directing the formation of the end products chlorophyll and heme (Tanaka and Tanaka, 2007). In darkness, the chlorophyll biosynthetic branch is blocked at the intermediate protochlorophyllide (Pchlide) because the conversion of Pchlide to chlorophyll is catalyzed by the light-dependent enzyme NADPH:protochlorophyllide oxidoreductase (POR) in plants (Runge et al., 1996; Su et al., 2001; Heyes and Hunter, 2005; Solymosi and Schoefs, 2010). However, the accumulation of excess free Pchlide and/or other pyrrole intermediates in darkness may produce reactive oxygen species (ROS) upon light irradiation and thereby cause cotyledon photobleaching or even cell death (Reinbothe et al., 1996; op den Camp et al., 2003; Wagner et al., 2004; Buhr et al., 2008). Therefore, chlorophyll biosynthesis is critical for plant survival and must be properly regulated, particularly during the switch from skotomorphogenesis to photomorphogenesis. Plants have evolved efficient mechanisms to regulate Pchlide content precisely in the dark.

Accumulating evidence shows that the biosynthetic pathway is primarily subject to transcriptional and posttranslational regulation (Tanaka and Tanaka, 2007; Mochizuki et al., 2010). ALA formation is viewed as a control point for regulation of chlorophyll supply. FLUORESCENT (FLU) was identified as an important regulatory protein that represses ALA synthesis by binding and thereby inhibiting the activity of glutamyl-tRNA reductase (GluTR) encoded by HEMA1 (Meskauskiene et al., 2001; Goslings et al., 2004). GluTRBP is the second GluTR binding protein that mediates spatial separation of ALA into heme biosynthesis (Czarnecki et al., 2011). Another regulator, GENOMES UNCOUPLED4 (GUN4), interacts with Mg-chelatase and stimulates its activity by facilitating substrate binding and/or product release (Larkin et al., 2003; Adhikari et al., 2011). These proteins are crucial for plant survival as a flu loss-of-function mutation is lethal during deetiolation or when plants are grown in light-dark cycles, whereas lack of GluTRBP is lethal and absence of GUN4 causes a complete absence of chlorophyll (Meskauskiene et al., 2001; Peter and Grimm, 2009).

Recently, several transcription factors have been found to play important roles in regulating chlorophyll biosynthesis during seedling deetiolation. The PHYTOCHROME-INTERACTING FACTORS (including PIF1, PIF3, PIF4, and PIF5) are a subset of the basic helix-loop-helix family of transcription factors and act as negative regulators of diverse phytochrome-mediated signaling responses (Leivar and Quail, 2011). In darkness, pit mutants display constitutive photomorphogenic phenotypes and overaccumulate Pchlide. Their etiolated cotyledons are severely photobleached after subsequent light illumination (Huq et al., 2004; Monte et al., 2004; Moon et al., 2008; Shin et al., 2009; Stephenson et al., 2009). It is believed that PIFs are negative regulators of chlorophyll biosynthesis in the dark and that light derepresses this response by triggering the proteasome-mediated degradation of PIFs (Al-Sady et al., 2006; Leivar et al., 2009; Shin et al., 2008; Shen et al., 2005). PIF1 directly regulates chlorophyll biosynthesis by binding the promoter of POR and activating its gene expression (Moon et al., 2008), whereas PIF3 indirectly regulates a number of biosynthetic genes, such as HEMA1 and GUN5 (Shin et al., 2009; Stephenson et al., 2009). It was also shown that ETHYLENE INSENSITIVE3 (EIN3) and its homolog EIN3-LIKE1 cooperate with PIF1 to inhibit Pchlide accumulation. EIN3 is able to upregulate POR and PORB gene expression directly. Consequently, their loss-of-function mutants accumulate excess Pchlide in etiolated seedlings and became photobleached when transferred to light (Zhong et al., 2009). A recent study demonstrated that DELLA proteins upregulate POR expression and limit the accumulation of ROS and photooxidative damage in PIF-dependent and -independent manners during seedling deetiolation (Cheminant et al., 2011). These studies together reveal that POR is an important target for transcriptional regulation in chlorophyll biosynthesis, consistent with its function in conversion of Pchlide into chlorophyllide during seedling deetiolation. However, the mechanism for direct regulation of Pchlide levels is still not understood.

In this study, we provide genetic, molecular and biochemical evidence to demonstrate that FHY3 and FAR1 redundantly promote chlorophyll biosynthesis by directly binding to and activating the expression of HEMB1, which encodes ALAD, with FHY3 playing a predominant role. We show that FHY3 physically interacts with the negative transcription regulator PIF1 to coordinate Pchlide synthesis and seedling greening. Furthermore, FHY3 expression is upregulated by white light during deetiolation. Our finding highlights FHY3 and FAR1 as positive and key transcription factors in directly regulating chlorophyll biosynthesis for seedling survival and provides insight into the functional divergence of these transposase-derived transcription factors in plants during evolution.

RESULTS

FHY3/FAR1 Promote Pchlide Accumulation in Etiolated Seedlings

A previous transcriptome analysis of Arabidopsis seedlings indicates that most of the genes involved in tetrapyrrole biosynthesis are highly responsive to light (Matsumoto et al., 2004). We speculated that there might exist a direct positive regulator(s) of Pchlide biosynthesis in etiolated seedlings. To this end, we tested some known transcription factors that have been demonstrated to be key components in the light signaling pathway (Jiao et al., 2007). Five-day-old etiolated seedlings of various mutants and the wild type were incubated with acetone overnight, and Pchlide levels were compared by scanning the
fluorescence emission in a fluorescence spectrophotometer (Huq et al., 2004). Consistent with previous studies, pif1 and pif3 mutants accumulated extremely high levels of Pchlide compared with the wild type (Huq et al., 2004; Moon et al., 2008; Shin et al., 2009; see Supplemental Figure 1 online). The elongated hypocotyl homolog (hyh), long hypocotyl in far-red1 (hfr1), and long after far-red light1 (laf1) mutants also had slight increased amounts of Pchlide. By contrast, the Pchlide content was decreased in the fhy3 mutant and was slightly reduced in the far1 and hy5 mutants compared with their corresponding wild-type seedlings (Figure 1A; see Supplemental Figure 1 online), suggesting that FHY3, FAR1, and HY5 may play positive roles in regulating chlorophyll biosynthesis in the dark. In this study, we focused on the roles of FHY3 and FAR1. We further found that the fhy3 far1 double mutant had even less Pchlide accumulation than their single mutant parents (Figure 1A), demonstrating a redundant function between these two proteins, with FHY3 playing a predominant role. In the following experiments, the function and mechanism of FHY3 will be investigated in more detail. To confirm whether low Pchlide in the fhy3-4 mutant is caused by the disruption of FHY3 protein, a FHY3p:FHY3 transgene in which the FHY3 open reading frame (ORF) is under the control of its own promoter was introduced into fhy3-4 (Lin et al., 2008). The transgene was found to complement the fhy3-4 mutant phenotype (Figure 1B). In addition, dexamethasone (DEX; 1 µM) treatment greatly restored the Pchlide level of fhy3-4/FHY3p:FHY3-GR transgenic plants (Lin et al., 2007) compared with mock-treated plants (Figure 1C), suggesting that nuclear targeting of FHY3 is required for its function. These results reveal that FHY3 and FAR1 promote Pchlide accumulation and likely function as positive regulators of chlorophyll biosynthesis in the dark.

Because the tetrapyrrole biosynthetic pathway separates into two different branches to produce heme and chlorophyll, we then tested if the heme branch was also affected by the fhy3 and far1 mutations. Noncovalently bound heme was extracted from 5-d-old etiolated seedlings and measured spectrophotometrically. However, no difference in heme production was observed between fhy3 far1, fhy3, and far1 mutants and the wild type (see Supplemental Figure 2 online).

**Loss of FHY3/FAR1 Enhances Seedling Greening during Deetiolation**

Next, we investigated how fhy3 and far1 mutations might influence the seedling deetiolation process. Etiolated seedlings grown in extended periods of darkness were illuminated with normal intensity white light (150 µmol m$^{-2}$ s$^{-1}$), and the percentage of green cotyledons (greening rate) was calculated after an additional 2 d. We found that the greening rate of fhy3-4 was much higher than that of the wild type after 4 d or longer of dark treatment. The far1-2 mutant showed a slightly increased greening rate compared with the wild type when grown in the dark for 6 d. Remarkably, the fhy3 far1 double mutant possessed strong greening ability, with a greening rate of nearly 75% even after 7 d of darkness (Figures 2A and 2B). Then we tested the greening process by transferring seedlings to various light intensities after 5 d of darkness. There were no distinguishable differences under low light conditions (<80 µmol m$^{-2}$ s$^{-1}$). However, when seedlings were transferred to light intensities of 150 µmol m$^{-2}$ s$^{-1}$ or higher, the greening rates of fhy3-4 and especially fhy3 far1 mutants were much higher than those of far1-2 and the wild type (Figure 2C). Most strikingly, when the etiolated seedlings were exposed to short periods of high light (1000 µmol m$^{-2}$ s$^{-1}$ for 5 h) followed by normal light (150 µmol m$^{-2}$ s$^{-1}$), 20 and 50% of fhy3-4 and fhy3 far1, respectively, turned green, whereas only 2% of far1-2 and <1% of wild-type seedlings survived (Figure 2D). In addition, DEX treatment resulted in a decrease in Pchlide accumulation compared with the mock-treated plants (Figure 1C).
greatly restored the greening phenotype of fhy3-4/FHY3p: FHY3-GR transgenic seedlings (Figure 2E). These data indicate that FHY3 and FAR1 play an important role in seedling greening during deetiolation.

Upon light illumination, excess Pchlide may generate ROS or free radicals, resulting in photobleaching or even cell death in the cotyledons (Reinbothe et al., 1996; Buhr et al., 2008). To test whether the increased greening rate of the mutants is the result of less photobleaching, we investigated ROS production by detecting 2′,7′-dichlorodihydrofluorescein diacetate (H₂DCFDA) fluorescence of the cotyledons (Zhong et al., 2009). We found that H₂DCFDA fluorescence was remarkably lower in the fhy3-4 and fhy3 far1 mutants and slightly lower in far1-2 than that in the wild type. However, chlorophyll autofluorescence was obvious in fhy3-4 and fhy3 far1 (Figure 2F). Furthermore, when stained with trypan blue (indicating dead cells), the cotyledons of the wild type and far1 were stained blue, whereas fhy3 and fhy3 far1 seedlings were barely stained (Figure 2G). Therefore, disruption of FHY3 and FAR1 prevents photobleaching and cell death.
FHY3 Activates HEMB1 Expression by Directly Binding to Its Promoter

The observed phenotypes prompted us to test whether the expressions of tetrapyrrole biosynthetic genes were influenced by FHY3 and FAR1 (see Supplemental Figure 3A online). fhy3 far1 double mutant and wild-type seedlings were grown in darkness for 5 d, and relative gene expression was analyzed by quantitative RT-PCR (qRT-PCR). We found that only a small set of genes, including HEMA3, HEMB1, FERROCHELATASE 2 (FC2), HEME OXYGENASE 1 (HO1), HO3, and HO4, were either up- or downregulated more than 1.5-fold in the fhy3 far1 double mutant compared with the wild type (see Supplemental Figure 3B online). It was previously shown that FHY3 activates downstream gene expression mainly through binding the FHY3/FAR1 binding site (FBS) (CACGCGC) present in promoters of its targets (Lin et al., 2007; Li et al., 2011). Therefore, we surveyed the promoter sequences of these tetrapyrrole biosynthetic genes and found that only HEMB1 contains a putative FBS motif, which is located 385 bp upstream of the ATG start code in reverse orientation (Figure 3A). To investigate whether FHY3 and FAR1 could bind to the HEMB1 promoter through this putative FBS motif, we first used a yeast one-hybrid system. GAD-FHY3 (fused with GAL4 activation domain) and GAD-FAR1 proteins were able to bind the wild-type HEMB1 oligonucleotide containing the FBS sequence (HEMB1wt:LacZ) and activate LacZ reporter gene expression, but they did not bind the mutant oligonucleotide (HEMB1m:LacZ, in which CCGCGTG was changed into GCttGTG) (Figure 3B). Next, we performed electrophoresis mobility shift assay (EMSA) to test whether FHY3 binds the HEMB1 promoter fragment in vitro. Our data showed that the FHY3 recombinant protein (N-terminal 250 amino acids of FHY3 fused with glutathione S-transferase; GST-FHY3) caused an upshift band with HEMB1 wild-type oligonucleotides labeled with $^32$P, and this band was abolished by excess unlabeled wild-type oligonucleotides but not by excess unlabeled mutant oligonucleotides (Figure 3C). To investigate further whether FHY3 binds HEMB1 DNA fragment in vivo, chromatin immunoprecipitation (ChIP) was performed using 35S:GUS-FHY3 (GUS for β-glucuronidase) transgenic seedlings (Wang and Deng, 2002). The ChIP DNA was quantified by real-time PCR with three sets of primers spanning the upstream promoter,
Figure 4B, the ALAD conversion ability was tested in vivo. As shown in isolated seedlings were fed exogenous 10 mM ALA for 12 h and far1 physiological response further, we grew the plants with or without fhy3 far1 and PBG production was determined (Vajpayee et al., 2000). We control (Figure 3D). Together, these results convalidate LUC reporter gene expression (Figure 3E). However, a point mutation in the transposase domain of FHY3 (FHY3-G305R; deficient in transcriptional activation; Lin et al., 2007) failed to activate LUC expression (Figure 3E). When LUC was driven by the HEMB1 promoter with mutations in the FBS motif, the expression level was drastically reduced and FHY3 was no longer able to activate it (Figure 3E). Next, using qRT-PCR analysis, we found that HEMB1 expression was modestly decreased in far1-2 and further dropped in fhy3-4, whereas it was significantly decreased in fhy3 far1 (Figure 3F), suggesting that FHY3 and FAR1 redundantly upregulate HEMB1 expression. In addition, DEX treatment also promoted HEMB1 expression in the FHY3p: FHY3-GR transgenic plants compared with the mock treatment (Figure 3G). We thus conclude that FHY3 and FAR1 positively regulate HEMB1 gene expression in plant cells.

FHY3/FAR1 Promote ALAD Activity

HEMB1 is one of two genes encoding ALAD, which catalyzes a reaction among the early steps of tetrapyrrole biosynthesis. We then examined how the ALAD protein level and enzymatic function were affected by fhy3 and far1 mutations. To this end, a polyclonal antibody against a peptide (amino acids 336 to 346) of ALAD was raised in rabbit. This antibody recognized the ALAD recombinant protein at a size of 55 kD, which is close to the predicted size of the ALAD mature protein, confirming the specificity of this antibody (see Supplemental Figure 4A online). Compared with wild-type plants, the ALAD protein level in far1-2 was slightly reduced, whereas it was clearly decreased in fhy3-4 and further reduced in fhy3 far1 double mutant seedlings (see Supplemental Figure 4B online), correlating well with the reduced HEMB1 transcripts in these mutants (Figure 3F).

To determine how ALAD function was affected, an in vitro enzymatic activity assay was performed in a reaction system containing both the plant protein extracts and ALA substrate, and PBG production was determined (Vajpayee et al., 2000). We found that less PBG was formed with proteins extracted from far1-2, and much less was formed from those of fhy3-4 and fhy3 far1 compared with the wild type (Figure 4A). Next, 5-d-old etiolated seedlings were fed exogenous 10 mM ALA for 12 h and the ALAD conversion ability was tested in vivo. As shown in Figure 4B, significantly less PBG was detected in the fhy3-4 and fhy3 far1 mutants than in the wild-type seedlings. To assess the physiological response further, we grew the plants with or without ALA feeding for 3 d and measured the Pchlide content as well as greening ability after light exposure. Without exogenous ALA treatment, Pchlide levels and greening rates were indistinguishable between fhy3 far1 and the wild type. However, when fed with ALA, wild-type seedlings contained drastically increased Pchlide, whereas fhy3 far1 accumulated only half that of the wild type (Figure 4C). Accordingly, almost all of the wild-type cotyledons were severely photobleached, whereas ~75% of the fhy3 far1 mutant seedlings were still able to turn green after light exposure (Figure 4D). These results firmly demonstrate that the ability to convert ALA into PBG in fhy3 and particularly fhy3 far1 mutants was reduced; therefore, FHY3 and FAR1 positively regulate ALAD function in plants.

Constitutive Expression of HEMB1 Rescues the fhy3 far1 Mutant Phenotypes

We then determined whether restoration of HEMB1 in the fhy3 far1 mutant could rescue its phenotype by constitutively expressing the HEMB1 ORF under the control of the cauliflower mosaic virus 35S promoter (35S:HEMB1). Multiple T1 resistant transgenic lines were obtained. Unexpectedly, all T2 transgenic lines segregated with nearly one-fourth of plants developing partially white leaves when grown in soil after 3 weeks (two lines were shown; Figure 5A). The HEMB1 transcripts in the transgenic lines were more than eightfold higher than those in the mutant (Figure 5B). These plants were eventually died and we were unable to obtain homozygous plants. Strikingly, we observed that the heterozygotes of HEMB1 overexpression plants rescued the Pchlide accumulation and seedling greening phenotypes of the fhy3 far1 mutant (Figures 5C and 5D), further confirming that the function of FHY3 and FAR1 in Pchlide synthesis and seedling greening is through the regulation of HEMB1. Consistent with this, HEMB1 is highly expressed in cotyledons during dark-to-light transition (see Supplemental Figure 5 online).

Overexpression, Absence, or Reduction of HEMB1 Impairs Plant Growth and Development

Similar to what was observed in the fhy3 far1 mutant background, the homozygous transgenic plants of 35S:HEMB1 overexpression lines in the wild type displayed photobleached leaves and did not survive (see Supplemental Figure 6 online). We speculated that HEMB1 might play essential role in regulating plant growth and development. To test this hypothesis, we obtained a T-DNA insertion mutant allele, Salk_016544, from the ABRC. This allele contains a T-DNA inserted into the eighth intron of HEMB1, and the insertion site was sequence confirmed, thereafter it was designated hemb1-1 (see Supplemental Figures 7A and 7B online). When the heterozygous progeny of hemb1-1 were grown on Murashige and Skoog (MS) plates containing 50 mg/mL kanamycin, nearly two-thirds (494 out of 747, P value > 0.75 by t test) of the seeds were resistant and developed normal seedlings. We attempted to use PCR genotyping to identify homozygotes from those kanamycin-resistant plants. However, all of the resistant plants were heterozygous, suggesting that the hemb1-1 mutation might be lethal in the
homozygous state. Then we dissected the developing siliques and scored the seeds under a dissecting microscope. In wild-type siliques, seeds were fully developed, whereas in 37 siliques from individual hemb1-1 heterozygous plants grown under the same conditions as the wild-type plants, 359 out of 1351 ovules were small, shrunken, and aborted (Figure 6A). The ratio of nonaborted seeds to aborted seeds was $3:1$ ($P$ value $> 0.1$). The lethality of the homozygous mutation was complemented by expressing HEMB1 (Figure 6A). Taken together, we conclude that hemb1-1 is a recessive embryo lethal mutation. To dissect precisely the stage of embryogenesis during which the hemb1 mutant arrests development, embryos within individual immature siliques from self-pollinated hemb1-1 plants were cleared and examined under a microscope. The normal embryos underwent typical developmental stages, ranging from preglobular, globular, heart, torpedo, and mature. However, the mutant embryos were arrested at the globular stage (see Supplemental Figure 7C online).

We then attempted to use RNA interference (RNAi) and artificial microRNA (amiRNA) transgenic approaches to knock down the endogenous HEMB1 expression in the Nossen wild-type background. When T1 seeds were germinated on 50 mg/L hygromycin plates, surprisingly, all of the HEMB1-RNAi (159 lines) and HEMB1-amiRNA (112 lines) resistant seedlings developed white or pale cotyledons. These seedlings did not develop true leaves even when grown in MS medium supplied with 2% Suc (Figure 6B). We collected these resistant seedlings and tested the HEMB1 expression by qRT-PCR, finding that the endogenous HEMB1 transcripts in those HEMB1-RNAi or HEMB1-amiRNA lines were severely reduced to <20% of those in the wild type (Figure 6C). These results revealed that severe reduction of HEMB1 mRNA causes seedling lethality. Taken together, our genetic data confirm that HEMB1 is critical for plant development and its transcript level has to be precisely maintained.

**FHY3 Physically Interacts with PIF1**

It has been shown that etiolated pif1 mutant seedlings over-accumulate Pchlide and are sensitive to photobleaching (Huq et al., 2004; Moon et al., 2008). Here, we found that the fhy3 and fhy3 far1 mutants display opposite phenotypes to those of pif1. To investigate how the two types of proteins, FHY3/FAR1 and PIF1, antagonistically regulate chlorophyll biosynthesis, we
constructed *fhy3 pif1* double and *fhy3 far1 pif1* triple mutants by genetic crossing. We found that the low Pchlide levels in the *fhy3* and *fhy3 far1* mutant seedlings were partially repressed by the *pif1* mutation in darkness (Figures 1A and 7A). When 4-d-old etiolated seedlings were exposed to light for 2 d, the greening rates of *fhy3 pif1* and *fhy3 far1 pif1* mutants were −26 and 70%, respectively, whereas the *pif1* mutant cotyledons were severely photobleached (Figure 7B). These results indicate that PIF1 partly suppresses the function of FHY3/FAR1 in regulating Pchlide accumulation and seedling greening.

It was then intriguing to ask whether there could be direct interaction between FHY3 and PIF1. The N-terminal domain of FHY3 has been shown to interact with HY5, CIRCADIAN CLOCK-ASSOCIATED1 (CCA1), and LATE ELONGATED HYPOCOTYL (LHY) proteins previously (Li et al., 2010; Li et al., 2011). We first performed a yeast two-hybrid assay using bait vector expressing the N-terminal 250 amino acids of FHY3 with the LexA DNA binding domain (LexA-FHY3N) and prey vector

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**Figure 5.** Constitutive Expression of HEMB1 Rescues the *fhy3 far1* Mutant Phenotypes.

(A) Images of *fhy3 far1/35S:HEMB1* homozygous transgenic plants (two lines are presented) showing white and bleached leaves when grown in soil for 3 weeks (16 h light/8 h dark). Bar = 1 cm.

(B) qRT-PCR showing high expression of HEMB1 in the transgenic lines. The expression levels were normalized to a UBQ endogenous control. Mean ± so from three biological replicates.

(C) Relative Pchlide levels of 5-d-old etiolated seedlings. WT, the wild type.

(D) Greening rate of 5-d-old etiolated seedlings following 2 d of light exposure. Mean ± so, n = 3.

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**Figure 6.** Absence or Reduction of HEMB1 Impairs Plant Growth and Development.

(A) A heterozygous *hemb1-1* mutant silique showing that approximately one-quarter of the embryos are aborted compared with the wild type (WT) and complemented transgenic siliques.

(B) Pale cotyledons of HEMB1-AmiRNA and HEMB1-RNAi transgenic seedlings grown on MS plates for 2 weeks. One representative line for each construct is shown. Bar = 0.5 cm.

(C) qRT-PCR showing extremely low expression of HEMB1 in the transgenic lines as in (B). The expression levels are normalized to an UBQ endogenous control. Mean ± so from three biological replicates.
expressing full-length PIF1 with the GAL4 activation domain (GAD-PIF1). Our data showed that LexA-FHY3N indeed interacts with GAD-PIF1 and the N-terminal domain containing the C2H2 zinc finger motif is responsible for mediating the interaction (Figure 7C). We further performed a pull-down analysis between His-tagged PIF1 and GST-fused FHY3N. The His-PIF1 proteins were incubated with immobilized GST or GST-FHY3N, and immunoprecipitated fractions were probed with an anti-His or anti-GST antibody. IB, immunoblot; IP, immunoprecipitation.

(E) Coimmunoprecipitation assay between GUS-FHY3 and TAP-PIF1 in vivo. Seedlings were grown in darkness for 4 d. After precipitation with anti-GUS antibody, proteins were immunoblotted with anti-GUS or anti-MYC antibodies. Arrow indicates GUS-FHY3 bands.

(F) LCI assay between FHY3N and PIF1 fused with the N-terminal or C-terminal fragment of firefly luciferase, respectively. Relative LUC activity is normalized to 35S:GUS internal control. Mean ± sd, n = 3.

(G) Relative HEMB1p:LUC reporter activity in Arabidopsis protoplasts cotransformed with the effector constructs. The relative LUC activities were normalized to the 35S:GUS internal control. Mean ± sd, n = 3. Protoplast transformation, incubation, and protein extraction were performed in darkness ([F] and [G]).

these data indicate that FHY3 physically interacts with PIF1 through its N-terminal domain.

As shown earlier, FHY3 activates HEMB1 expression in plant cells; we then ask whether FHY3-PIF1 interaction could influence HEMB1 expression. To this end, we cotransformed FHY3 (35S: FHY3) and/or PIF1 (35S:PIF1) effectors together with HEMB1p:LUC reporter construct in Arabidopsis protoplasts. Our data showed that PIF1 alone inhibited the transcription of the LUC reporter gene. Coexpression of PIF1 largely suppressed the activation activity of FHY3 on the HEMB1p:LUC reporter (Figure 7G). Consistent with this, the HEMB1 transcript level in fhy3 far1 mutant was largely derepressed by the pif1 mutation (see Supplemental Figure 8B online). These results demonstrate that PIF1 interferes with FHY3/FAR1-activated HEMB1 transcription.

FHY3 Is Upregulated by White Light

To assess how expressions of FHY3 and FAR1 themselves are regulated during deetiolation, 4-d-old etiolated wild-type seedlings were transferred to white light in a time course. Real-time
RT-PCR analysis showed that the FHY3 transcript level was elevated after light treatment for 6 h, whereas FAR1 gene expression was slightly affected by light (Figure 8A). To investigate the regulation of FHY3 protein, dark-grown 35S:GUS-FHY3 transgenic seedlings were exposed to light and GUS activity was determined. We found that the GUS activity was increased along with light treatment (Figure 8B). Moreover, by bioluminescence analysis of the FHY3p:FHY3-LUC reporter gene, we further showed that the FHY3 level was increased in white light (see Supplemental Figure 9A online). Consistent with being the direct target of FHY3/FAR1, the HEMB1 transcript was induced by light partly dependent on FHY3/FAR1. Its protein content was also increased in wild-type plants upon light illumination (see Supplemental Figures 9B and 9C online). These findings indeed support the positive role of FHY3 in regulating chlorophyll biosynthesis.

DISCUSSION

FHY3/FAR1 Are Key Components That Positively Regulate Chlorophyll Biosynthesis

The tetrapyrrole biosynthetic pathway is finely regulated at multiple levels, and some negative regulators have been reported in plants (Tanaka and Tanaka, 2007). In this study, we provide an array of evidence to show that two transposase-derived transcription factors, FHY3 and FAR1, positively regulate chlorophyll biosynthesis through directly activating HEMB1 gene expression. First, yeast one-hybrid, EMSA and ChIP experiments demonstrate that FHY3 protein directly binds to the promoter of HEMB1 and that the typical FBS cis-element in the promoter is responsible for mediating the binding. Second, FHY3 activates HEMB1 transcription in an FBS motif-dependent manner in protoplast transient expression assays. Third, HEMB1 gene expression in vivo and in vitro ALAD catalytic activities are reduced by fhy3 and far1 mutations (Figures 3 and 4). Consistent with these molecular data, fhy3, far1, and fhy3 far1 mutants accumulate less Pchlide than the wild type in darkness (Figure 2). In the tetrapyrrole biosynthetic pathway, HEMB1 is a unique gene that not only has a typical FBS motif in its promoter but also belongs to a small set of genes that are regulated by FHY3 and FAR1. Our study indeed suggests that HEMB1 defines a key direct target of FHY3 and FAR1 for transcriptional activation. It should be noted that HEMC and CHLG were reported as putative FHY3 targets by a ChIP sequence approach (Ouyang et al., 2011). We find that FHY3 and FAR1 have minor effects on the expression of HEMC and CHLG (see Supplemental Figure 3 online). The discrepancy could be due to the different transgenic plants (35S:3FLAG-FHY3-3HA in Ouyang et al. [2011] and 35S:GUS-FHY3 in this study) and growth conditions used in the two studies.

Seedling survival during the transition from skotomorphogenesis to photomorphogenesis is important for land plants particularly under light stress environments. Targeting genes in the early steps of the biosynthetic pathway for regulation might provide seedlings potential advantages because excess accumulation of photosensitizers (such as Pchlide) of the later steps may generate ROS upon initial light exposure (Tanaka and Tanaka, 2007; Solymosi and Schoefs, 2010). The size of the Pchlide pool must be stoichiometrically linked to the amount of POR enzymes (Runge et al., 1996; Buhr et al., 2008). Thus, Pchlide content in darkness has to be maintained at relatively low level in preparation for rapid transition. ALA formation is the rate-limiting step of the entire pathway, and GluTR activity is tightly regulated (Mochizuki et al., 2010). For example, failure to repress GluTR activity in the dark results increased accumulation of Pchlide in the flu mutant (Goslings et al., 2004). HEMB1 functions just after ALA formation among the common steps in the tetrapyrrole biosynthetic pathway. Our data quantitatively and collectively demonstrate that different degrees of Pchlide accumulation (Figure 1A) and photobleaching (Figure 2) phenotypes correlate with the transcript level of HEMB1 (Figure 3F), ALAD protein level (see Supplemental Figure 4B online), and enzyme activity (Figures 4A and 4B) in the far1, fhy3, and fhy3 far1 mutants. On the other hand, the endogenous HEMB1 level might be subject to fine regulation (see below in detail). We thus
believe that a low-fold gene expression change of HEMB1 is likely sufficient to trigger the drastic seedling greening phenotype in the fhy3 far1 mutant. In Arabidopsis, ALAD is encoded by HEMB1 and HEMB2. HEMB1 is greatly induced in cotyledons during dark-to-light transition and expressed in all tissues and developmental stages examined. However, the expression of HEMB2 is barely detected (see Supplemental Figure 5 online), suggesting that HEMB1 is the major contributor at this step. This notion is further supported by genetic study showing that constitutive expression of HEMB1 rescues the fhy3 far1 mutant phenotypes (Figure 5). A previous study showed that FHY3 and FAR1 regulate chloroplast development through activating ARC5 expression (Ouyang et al., 2011). However, an arc5 loss-of-function mutant did not show differences in Pchlide level or greening ability relative to Landsberg erecta wild type (see Supplemental Figure 10 online), indicating that ARC5 is not involved in the regulation of chlorophyll biosynthesis during deetiolation.

Most strikingly, FHY3 expression is repressed in darkness, and light induces its expression probably through activating phytochrome signaling. Therefore, we propose a model in which, in darkness, less HEMB1 is induced by FHY3 (and FAR1), resulting in small pool of Pchlide. After light irradiation, increased FHY3 levels activate HEMB1 expression, thereby promoting the conversion of ALA to PBG and subsequent Pchlide accumulation for chlorophyll synthesis and photoautotrophic growth (Figure 9).

When excess Pchlide accumulates in the dark-grown seedlings, POR represents a critical regulatory layer for the control of chlorophyll biosynthesis during seedling greening. In agreement with this, ga1-3 and gai mutant seedlings showed increased POR expression and were more resistant to photooxidative damage, despite a high accumulation of Pchlide in the dark (Cheminant et al., 2011). Moreover, overexpression of POR promoted seedling greening (Sperling et al., 1997; Cheminant et al., 2011). By contrast, reduction of POR expression in the pif1 and ein3 mutants resulted in severe photobleaching during dark-to-light transition (Moon et al., 2008; Zhong et al., 2009).

Interestingly, we observed that mutations in fhy3 and far1 do not alter the nonbound heme content, probably through opposite regulation of FC2, HO1, HO3, and HO4 genes that act in the heme branch. Thus, it is postulated that FHY3 and FAR1 specifically contribute to the chlorophyll biosynthetic pathway. This is in agreement with the idea that newly synthesized tetrapyrroles need to be directed to the chlorophyll branch rather than the heme branch during initial seedling greening (Stephenson and Terry, 2008).

**Integration of Two Distinct Types of Transcription Factors in Chlorophyll Biosynthesis Regulation**

The discovery of positive regulators is of utmost significance for understanding the exquisite regulation of the chlorophyll biosynthetic pathway. In this study, we find that by means of its N-terminal domain FHY3 physically interacts with PIF1, a phytochrome-interacting transcription factor (Figure 7). The pif1 mutant accumulates extremely high levels of Pchlide in the dark, and PIF1 has been revealed as a negative regulator of chlorophyll biosynthesis (Huq et al., 2004; Moon et al., 2008). Although PIF1 may indirectly regulate GUN5 expression (Shin et al., 2009), the mechanism of PIF1 in regulating Pchlide synthesis is not well understood. We show that PIF1 directly interacts with FHY3 (and maybe FAR1 as well) and partly represses FHY3 activation activity on HEMB1 gene expression. It is speculated that the regulation of PIF1 in Pchlide synthesis in the dark may be partly dependent on the function of FHY3/FAR1. Accordingly, the fhy3 far1 double mutant is largely able to suppress the pif1 mutant phenotypes (Figure 7). Thus, two distinct types of transcription factors in the phytochrome signaling pathway coordinate to regulate an important biological response. In agreement with this finding, a recent analysis reported that FHY3 and PIF1 (PIL5) coregulate more than 100 genes (Ouyang et al., 2011). They could also be involved in mediating other diverse processes through their interaction. Our finding supports the hypothesis that the expression of genes involved in chlorophyll biosynthesis is regulated by a tight and complex mechanism comprising multiple components (Tanaka and Tanaka, 2007). It also implies that both positive and negative players work coordinately to optimize chlorophyll biosynthesis, thus enabling etiolated seedlings to prepare properly for emerging at the soil surface (Figure 9).

We also noticed that HY5 likely has a positive role in regulating Pchlide synthesis (see Supplemental Figure 1 online). HY5 acts as a key player in light signaling and interacts with FHY3 and FAR1 (Oyama et al., 1997; Li et al., 2010). It will be
interesting to investigate whether FHY3, PIF1, and HY5 work together to coregulate chlorophyll biosynthesis in plants. Recent studies also demonstrated the involvement of HY5/FAR1 in mediating phyA signaling homeostasis and the circadian clock by cooperating with negative transcription factors, such as HY5, CCA1, and LHY (Li et al., 2010; Li et al., 2011). Hence, integration with a specific transcription repressor(s) in regulating a distinct biological process likely defines a common molecular mechanism underlying HY5/FAR1 action.

HEMB1 Is Critical for Plant Development

Although HEMB1 has been cloned from some species (Kaczor et al., 1994; Polking et al., 1995), its function in plant development has not been demonstrated. Our study collected genetic evidence suggesting that besides being a key gene for chlorophyll biosynthesis, HEMB1 is critical for plant embryonic and postembryonic development. The T-DNA insertion mutation in HEMB1 is embryo lethal, and the HEMB1 RNAi and amiRNA transgenic plants display seedling lethality after germination (Figure 6). Other studies have implied that a large number of plastid-targeted proteins are required for proper embryogenesis in Arabidopsis (Hsu et al., 2010; Bryant et al., 2011). However, no direct evidence has shown that genes in chlorophyll biosynthesis are involved in regulating embryoid development. We speculate that the arrested embryo development of the hemb1 mutant might be caused by disruption of embryo pigmentation and/or the absence of an essential intermediate(s) of the tetrapyrrole biosynthetic pathway. Moreover, homozygotes of HEMB1 overexpression lines in either wild-type or fhy3 far1 mutant backgrounds develop white leaves and are photobleached when grown in soil (Figure 5; see Supplemental Figure 6 online). These data firmly indicate that HEMB1 is crucial for plant development and its regulation is mainly at the transcriptional level. Consistent with this notion, both Pchlide levels and seedling greening rates correlate well with the HEMB1 transcript levels in the far1, fhy3, and fhy3 far1 mutants (Figures 2 and 3F). When the endogenous HEMB1 mRNA drops to <20% of the wild type, the RNAi or amiRNA transgenic plants develop only white cotyledons and are not able to survive (Figure 6). Therefore, it is likely that a certain threshold level of HEMB1 transcript is required for embryo and young seedlings to ensure their viability. In agreement, HEMB1 is expressed at a relatively high level in the aboveground tissues examined (see Supplemental Figure 5 online).

ALAD is an ancient and conserved protein not only in plants and fungi but also in animals and humans. The deficiency of ALAD in humans results in increased excretion of ALA in the urine, leading to ALAD-deficient porphyria, an autosomal recessive disorder (Maruno et al., 2001). Thus, ALAD activity in neonatal blood is used as a screening tool for ADP (Schulze et al., 2001). The human ALAD gene has two promoter regions that generate different transcripts by alternative splicing, although the enzyme produced is identical, suggesting that it is regulated transcriptionally and posttranscriptionally (Schubert et al., 2009). It is assumed that the functionality and regulatory mechanism of ALAD might be conserved in organisms.

Genetic studies have revealed that some other proteins involved in tetrapyrrole biosynthesis are also essential for plant growth and development. For example, plants with antisense inhibition of HEMA1 lack chlorophyll and fail to survive under normal growth conditions (Kumar and Söll, 2000). Arabidopsis and tobacco (Nicotiana tabacum) transgenic etiolated plants with overexpression of HEMA1 accumulate excessive Pchlide and do not survive after subsequent illumination (Schmied et al., 2011). Absence of GluTRBP is lethal, and mutants with reduced GluTRBP content are photobleached under high light stress (Czarnecki et al., 2011). Similar to HEMB1, overexpression of GUN5, a gene encoding the ChiK subunit of Mg$^2+$-chelatase, causes spontaneous photobleaching and cell death (Shin et al., 2009). However, unlike hemb1, the gun5 loss-of-function mutant has a pale phenotype and is able to grow in soil (Mochizuki et al., 2001). Therefore, the functionalities of these genes may be overlapping as well as distinct, although they are all required for tetrapyrrole biosynthesis in plants.

METHODS

Plant Materials and Growth Conditions

The fhy3-4, far1-2, and fhy3 far1 mutants are of Arabidopsis thaliana Nossen ecotype (Hudson et al., 1999; Wang and Deng, 2002; Lin et al., 2007). The hemb1-1 (Salk_016544), pif1-2 (Huq et al., 2004), hy5 (Oyama et al., 1997), hyh (csb49765; Kleine et al., 2007), hfr1 (Yang et al., 2005), pif3 (Salk_030753; Kim et al., 2003) and lfr1 (Salk_009403G) mutant are in the Columbia (Col) ecotype. The arc5 mutant is of the Landsberg erecta ecotype (Ouyang et al., 2011). SS5:GUS-FHY3 (Wang and Deng, 2002), FHY3p:FHY3-GFP, FHY3p:FHY3 (Lin et al., 2007), and FHY3p:FHY3-LUC (Li et al., 2011) are transgenic lines in the fhy3-4 mutant background. Double and triple mutants/transgenic plants were generated by genetic crossing. Homozygous lines were confirmed by genotyping or sequencing. After sterilization, seeds were sown onto MS medium containing 1% Suc and 0.8% agar and were incubated at 4°C in darkness for 3 d, followed by irradiation for 9 h with white light to promote uniform germination.

Greening Rate Measurement

Dark-grown seedlings were transferred to continuous white light (supplied with light-emitting diodes) for 2 d at 22°C or otherwise as indicated in the text. Greening rate was determined by counting the percentage of dark-green cotyledons from 50 to 80 seedlings of each genotype. At least three independent biological repeats were performed.

Pchlide Determination and Fluorescence Imaging of ROS

For Pchlide measurement, 50 seedlings were grown in darkness for 4 or 5 d. The samples were homogenized in 500 μL of ice-cold 80% acetone and incubated in darkness for 4 h. After centrifugation at 5000g for 5 min, 150 μL of the supernatant was mixed with 350 μL of glycol. Room temperature fluorescence was excited at 440 nm and scanned from 600 to 700 nm by a fluorescence spectrophotometer (Hitachi). For fluorescence imaging of ROS, seedlings were incubated with 10 μM H$_2$DCFDA in 10 mM Tris-HCl, pH 7.2, for 10 min as described (Joo et al., 2005). H$_2$DCFDA and chlorophyll fluorescence images were captured by DMi4500 fluorescence microscope equipped with a charge-coupled device camera (Leica).

Trypan Blue Staining

Seedlings were boiled for 5 min in staining solution (1.8 mL phenol, 2 mL lactic acid, 2 mL glycerol, and 2 mL of 1 mg/mL trypan blue stock solution) and stained overnight. Tissues were mounted on slides and photographed on a dissecting microscope.
Phenotypic Analysis of Embryo Development

Embryos were excised from siliques at different developmental stages and cleared with Herr’s solution (Herr, 1971) overnight at 37°C. Samples were mounted between a microscope slide and a cover slip with a drop of Hoyer’s solution (7.5 mL water, 1.3 g glycerol, 1.9 g gum arabic crystals and 25 g chloral hydrate) and observed with a microscope (Olympus).

Plasmid Construction

To generate LacZ reporter genes driven by the HEMB1 promoter with a wild-type or mutant FBS motif, the 39-bp oligonucleotides were synthesized as two complementary primers (HEMB1WF and HEMB1WR for the wild type, and HEMB1MF and HEMB1MR for mutant) with an EcoRI site overhang at the 5’ end and an XhoI site overhang at the 3’ end, respectively. The annealed oligonucleotides were ligated into the EcoRI-XhoI sites of pLaczZ014 (Lin et al., 2007), resulting in HEMB1wtLacZ and HEMB1mutLacZ, respectively.

To produce a LUC reporter gene driven by the HEMB1 promoter, a 1.8-kb fragment upstream of HEMB1 ATG translational start codon was PCR amplified with primers HEMB1F and HEMB1P2 from Col genomic DNA. The PCR fragment was inserted into the pGEM-T Easy (Promega) vector to produce pGEM-HEMB1p. To mutagenize the FBS motif (GGCGCGT) in the HEMB1 promoter, the fragment was amplified from pGEM-HEMB1p template using primers HEMB1pm1 and HEMB1pm2 in which the FBS motif site was changed into GCttGTG, giving rise to pGEM-HEMB1pm. After sequencing confirmation, the wild-type and mutant fragments were released from pGEM-HEMB1p and pGEM-HEMB1pm cut by HindIII and BamHI and ligated into the HindIII-BamHI site of pYY96 vector (Yamamoto et al., 1998) to produce HEMB1p: LUC and HEMB1pm:LUC, respectively.

To obtain the HEMB1 cDNA clone, the first-strand cDNA was reverse transcripted using oligo(dT) primer from total RNA extracted from Col wild-type seedlings. The ORF of HEMB1 gene was amplified with primers HEMB1F and HEMB1R by high-fidelity Pfu DNA polymerase (Invitrogen) and cloned into the pGEM-T Easy vector, resulting in pGEM-HEMB1.

To construct the HEMB1 overexpression binary vector, the HEMB1 gene was released from pGEM-HEMB1 by digestion with NcoI and BglII and ligated into the NcoI-BglII site of pCAMBIA1302 (http://www.cambia.org/daisyl/cambia/SBS/) to produce 3SS:HEMB1. In addition, the HEMB1 promoter was released from pGEM-HEMB1p cut by SalI and SacI and ligated into the SacI-SalI site of 3SS:HEMB1 to replace the 3SS promoter, resulting in HEMB1p:HEMB1.

To generate an RNAi construct for HEMB1, a 540-bp conserved cDNA fragment of HEMB1 was PCR amplified with primers HEMB1R1 (containing SpeI and KpnI sites at the 5’ end) and HEMB1R2 (containing SacI and BamHI sites at the 5’ end) from pGEM-HEMB1 plasmid DNA. The PCR fragment was cloned into pGEM-T Easy vector, resulting in pGEM-HEMB1R. The KpnI-BamHI fragment was released from pGEM-HEMB1R and ligated into the KpnI-BamHI site of pDS1301 (Yuan et al., 2007) to produce pDS1301-KB. Then, the SpeI-SalI fragment was released from pGEM-HEMB1R and ligated into the SpeI-SalI site of pDS1301-KB to produce pDS1301-HEMB1-RNAi.

To make the HEMB1 amiRNA construct, the amiRNA target sequence of HEMB1 (5’-TAAAGACACTGTTTACCCCAAC-3’) and primers HEMB1A1, HEMB1A2, HEMB1A3, and HEMB1A4 were designed using the WMD3 Web microRNA Designer (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi; Schwab et al., 2006). These primers were used to amplify the amiRNA precursor by overlapping PCR from the pRS300 template to produce the fragment containing HEMB1 target amiRNA foldback. The amiRNA foldback was released with KpnI and SpeI and then ligated to the KpnI-SpeI site of pDS1301 for constitutive expression under the control of the cauliflower mosaic virus 3SS promoter, resulting in pDS1301-HEMB1-amiRNA.

To construct a HEMB1 bacterial expression vector, a 1.13-kb (from amino acid 53 to 430, without the putative chloroplast transit signal peptide) cDNA fragment of HEMB1 was PCR amplified with primers HEMB1B1 and HEMB1B2 from pGEM-HEMB1. The PCR fragment was released with SacI and SalI and then ligated to the SacI-SalI site of pET28a to produce the pHEMB1-6His.

To construct PIF1 bacterial expression and yeast one-hybrid vectors, the PIF1 ORF was PCR amplified with primers PIF1-F and PIF1-R from Col cDNA and cloned into pGEM-T Easy vector, resulting in pGEM-PIF1. The PCR fragment was released with EcoRI and SalI, then ligated to the EcoRI-SalI site of pET28a to produce p6His-PIF1, and ligated to the EcoRI-XhoI site of JG4-5 (Clontech) to produce GAD-PIF1, respectively.

The yeast vectors LexA-FHY3N, GAD-FHY3, and GAD-FAR1, the recombinant protein construct GST-FHY3N, and the transient expression vectors pSPYCE-FHY3 and pSPYCE-FHY3-G305R were described previously (Lin et al., 2007).

To construct LCI vectors for FHY3 and PIF1, full-length FHY3 and FHY3N were released from pGEM-FHY3 or pGEM-FHY3N by digestion with BamHI and SalI and cloned into the BamHI-SalI site of 3SS:NLuc (Chen et al., 2008) to produce FHY3-NLuc and FHY3N-NLuc, respectively. PIF1 was released from pGEM-PIF1 cut with KpnI and SalI and inserted into the KpnI-SalI site of 3SS:CLuc to generate CLuc-PIF1.

All primer sequences are listed in Supplemental Table 1 online.

Yeast Assays

Yeast hybrid assays were performed as previously described (Lin et al., 2007). Briefly, for yeast one-hybrid assays, the GAD fusion constructs were cotransformed with the LacZ reporter plasmids. Transformants were grown on SD/-Trp-Ura dropout liquid medium, and relative β-galactosidase activity was quantified by a spectrophotometer. For yeast two-hybrid assays, the respective combinations of GAD and LexA fusions were cotransformed into the yeast strain EGY48, which contains the LexAop-LacZ reporter construct (Clontech). Transformants were grown on SD/-Trp-Ura-His dropout plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside for blue color development.

Purification of Recombinant Protein

GST, GST-FHY3N, His-PIF1, and His-HEMB1 recombinant fusion proteins were induced by isopropyl-β-D-1-thiogalactopyranosidase and expressed in the Escherichia coli BL21 (DE3) strain. The proteins were then purified by Glutathione Sepharose 4B beads (GE Healthcare; for GST and GST-FHY3N) or Ni-NTA Agarose (Qiagen; for His-PIF1 and His-HEMB1) following the manufacturer’s instructions.

EMSA

EMSA analysis was performed as previously described (Lin et al., 2007). Briefly, HEMB1 complementary oligonucleotides were labeled with [γ-32P]dATP and incubated with GST-FHY3N or GST proteins in the absence or presence of unlabeled oligonucleotides followed by separation on polyacrylamide gels. The DNA–protein binding signal was exposed to x-ray film and developed. The oligonucleotide sequences are shown in Supplemental Table 1 online.

Antibody Production and Immunoblotting

The peptide corresponding to amino acids 336 to 346 of ALAD (EAR-EDAEAGAD) conjugated with KLH was synthesized (Cali-Bio), and a polyclonal antibody was raised in rabbit. For immunoblotting, seedlings were homogenized in extraction buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl2, 0.1% Tween 20, 1 mM PMSF, and 1
complete protease inhibitor cocktail (Roche). The extracts were centrifuged at 14,000g twice at 4°C for 10 min each, and protein concentration was determined by Bradford assay (Bio-Rad). Proteins were boiled in SDS loading buffer, separated by 10% SDS-PAGE gels, and blotted onto polyvinylidene fluoride membranes (Pall). The proteins were then incubated with anti-ALAD (1:1000 dilution) or anti-His (Abcam) primary antibodies and subsequently the horseradish peroxidase–conjugated goat-anti-rabbit secondary antibody (Abcam). The protein bands were visualized by the standard ECL method.

Pull-Down Assay

About 2 μg of purified recombinant bait proteins (GST-FHY3N and GST) and 2 μg of prey proteins (His-PiF1) were incubated in binding buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.6% Triton X-100) for 2 h at 4°C. Glutathione Sepharose 4B beads were added and incubated for 1 h. After washing with binding buffer, precipitated proteins were eluted in 2× SDS loading buffer. The proteins were then size fractioned on 10% SDS-PAGE and immunoblotted by anti-His or anti-GST antibodies (Abcam).

Coimmunoprecipitation

For coimmunoprecipitation assays, seedlings were grown in the dark followed by treatment with 50 μM MG132. Total proteins were extracted with extraction buffer and incubated with 2 μg anti-GUS (Invitrogen) antibody for 2 to 3 h at 4°C. Fifty microliters of protein G-Sepharose (Roche) was added and incubated for another 2 to 3 h. The sepharose beads were washed three times with coimmunoprecipitation buffer, and the precipitated proteins were eluted in 2× SDS loading buffer by boiling for 10 min. The proteins were separated on 10% SDS-PAGE gel and detected by immunoblotting using anti-MYC (Abcam) and anti-GUS antibodies.

ChIP

The 35S:GUS-FHY3 transgenic plants grown in darkness for 5 d were used for ChIP assays following the procedure as described (Leibfried et al., 2005). Briefly, the seedlings were cross-linked with 1% formaldehyde and ground to powder under liquid nitrogen. After isolation and sonication, the chromatin complexes were incubated with anti-GUS antibody (Invitrogen) or anti-FLAG antibody (Abcam) as a negative control. The precipitated DNA fragments were recovered and quantified by real-time PCR with primers shown in Supplemental Table 1 online.

LUC Activity Assay

Protoplast isolation and transient expression assays were performed as described previously (Lin et al., 2007). For transient expression assays, the reporter plasmids (HEMB1p::LUC or HEMB1pm::LUC), effector constructs (pSPYCE::FHY3 and pSPYCE::FHY3-G305R), and 35S:GUS internal control were cotransformed into protoplasts. For LCI assays, plastid combinations of various N- and C-terminal LUC fusions were cotransformed with 35S::GUS internal control. The protoplasts were pelleted and resuspended in 1× cell culture lysis reagent (Promega). The GUS fluorescence was measured using a Modulus luminometer/fluorometer with a UV fluorescence optical kit (Promega). The LUC activity was detected with a luminescence kit using LUC assay substrate (Promega). The relative reporter gene expression levels were expressed as the LUC/GUS ratios.

RNA Extraction and qRT-PCR

Seeds were treated as indicated in the text, and plant total RNA was extracted by RNA extraction kit (Tiangen). The first-strand cDNA was synthesized by reverse transcriptase (Invitrogen). Real-time PCR was performed with the SYBR Premix ExTaq kit (Takara) in a 15-μL reaction system following the manufacturer’s instructions. Three biological replicates were performed for each sample, and the expression levels were normalized to those of UBO. All primers sequences are listed in Supplemental Table 1 online.

In Vivo ALA Feeding

For phenotype analysis, the seedlings were grown on MS plates containing 100 μM ALA (Sigma-Aldrich) in darkness for 4 d. For ALAD activity assay, the dark-grown seedlings were transferred to 10 mM ALA solution (5 mM MgCl2 and 10 mM NaH4PO4, pH 7.0) under green light and incubated for 12 h.

ALAD Enzyme Activity Determination and PBG Measurement

The in vitro ALAD activity was determined as Vaipaeae et al. (2000) described with minor modifications. Briefly, tissues (~0.2 g) were homogenized with 1 mL extraction buffer (50 mM Tris-HCl, pH 8.2, and 0.1 mM DTT) in a prechilled mortar and pestle. The homogenate was filtered through four layers of cheese cloth, and the filtrate was centrifuged at 10,000g for 1 h at 4°C. The supernatant was used for ALAD activity assay. One milliliter of the filtrate was incubated with reaction buffer (0.27 mL of 1 mg/mL ALA, 1.35 mL of 50 mM Tris-HCl, pH 8.2, 0.1 mM DTT, and 0.08 mL of 0.2 M MgCl2) for 2.5 h at 37°C. The reaction was terminated by the addition of 0.3 mL of 3.0 M trichloroacetic acid. After cooling, samples were centrifuged at 2000g for 10 min and used for PBG determination. The ALAD activity is expressed as nmol of PBG formed/mg protein/h at 37°C.

PBG content determination was performed as described (Mauzerall and Granick, 1956; Kayser et al., 2005). Briefly, samples were homogenized and resuspended in 1 mL of 0.15 M cold trichloroacetic acid. After centrifugation at 50,000g for 30 min at 0°C, the supernatants were adjusted to a pH of ~5.5 by addition of 1 N NaOH and 0.5 M sodium acetate. They were passed through a Dowex 1×8 column (200 to 400 mesh; Sigma-Aldrich) equilibrated at pH 4.6 to absorb PBG. The resins were washed eight times with double distilled water, and PBG was eluted two times with 0.4 mL of 1 M acetic acid. The eluates were mixed with an equal volume of Ehrlich reagent, and absorbance of the mixture was determined at 555 nm after 10 min on a spectrophotometer. PBG content of the samples was calculated using a standard curve generated by commercial PBG (Sigma-Aldrich).

Heme Determination

The content of noncovalently bound heme was measured according to the method of Richter et al. (2010). Briefly, tissues were homogenized, and noncovalently bound heme was extracted with 5 mL of extraction solution (2 mL of dimethyl sulfoxide, 10 mL of acetone, and 0.5 mL of 37% HCl) followed by centrifugation at 16,000g for 10 min. Heme was transferred to ether by addition of 3 mL of diethyl ether, 2 mL of saturated NaCl, and 10 mL of water. The ether phase was mixed with ethanol and flowed through a DEAE-Sepharose CL-6B column (GE Healthcare). After sequential washing with diethyl ether:ethanol (3:1, v/v), diethyl ether:ethanol (1:1, v/v), and ethanol, heme was eluted with ethanol:acetic acid:water (81:9:10, v/v/v) and quantified spectrophotometrically at 398 nm using the extinction coefficient of 144 M−1 cm−1.

Arabidopsis Transformation

The plant binary expression vectors 35S:HEMB1, HEMB1p::HEMB1, pDS1301-HEMB1-RNAi, or pDS1301-HEMB1-amiRNA were electroporated into the Agrobacterium tumefaciens strain GV3101 and then introduced into the wild type or fhy3 fai1 via the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on MS plates in the presence of 50 mg/L hygromycin.
Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL data libraries under the following accession numbers: FHY3 (At3g22170), FAR1 (At4g15090), HEMB1 (At1g69740), PIF1 (At2g20180), UBQ (At4g52590), HEMA1 (At1g8290), HEMA2 (At1g09940), HEMA3 (At2g31250), GSA1 (At5g93570), GSA2 (At3g48730), HEMB2 (At1g4318), HEMC (At5g08280), HEMD (At2g26540), HEME1 (At3g14930), HEME2 (At2g40490), HEMF1 (At1g03475), HEMF2 (At4g32050), CPO2 (At5g52300), HEMG1 (At4g01690), HEMG2 (At5g14220), FC1 (At5g26030), FC2 (At2g30390), H01 (At2g26670), H02 (At2g26550), H03 (At1g69720), H04 (At1g58300), HY2 (At3g09150), CHLD (At1g08520), CHLH (At5g13630), CHL1 (At4g18480), CHL2 (At5g45930), CHLM (At2g25080), CRD1 (At3g56940), PORA (At5g54190), PORB (At4g27440), PORC (At1g03630), DVR (At5g18660), CAO (At1g44446), and CHLG (At3g51820).

Supplemental Data

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

Supplemental Figure 1. Relative Phcthile Fluorescence of Various Mutants and the Wild Type.

Supplemental Figure 2. Noncovalently Bound Heme Contents in fhy3, far1, and fhy3 far1 Mutants and the Wild Type.

Supplemental Figure 3. Expression of Genes Involved in the Tetrapyrrole Biosynthetic Pathway.

Supplemental Figure 4. Confirmation of ALAD Antibody and Protein Level.

Supplemental Figure 5. HEMB1 and HEMB2 Expression Patterns in Different Stages and Tissues.

Supplemental Figure 6. Phenotype of HEMB1 Overexpression Plants.

Supplemental Figure 7. Identification and Characterization of hemb1 Mutant.

Supplemental Figure 8. Interaction between FHY3 and PIF1.

Supplemental Figure 9. FHY3 and HEMB1 Are Induced by Light.

Supplemental Figure 10. Characterization of the arc5 Mutant Phenotype.

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

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