Photoactivated phytochromes interact with HEMERA and promote its accumulation to establish photomorphogenesis in *Arabidopsis*

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Plant development is profoundly regulated by ambient light cues through the red/far-red photoreceptors, the phytochromes. Early phytochrome signaling events include the translocation of phytochromes from the cytoplasm to subnuclear domains called photobodies and the degradation of antagonistically acting phytochrome-interacting factors (PIFs). We recently identified a key phytochrome signaling component, HEMERA (HMR), that is essential for both phytochrome B (phyB) localization to photobodies and PIF degradation. However, the signaling mechanism linking phytochromes and HMR is unknown. Here we show that phytochromes directly interact with HMR to promote HMR protein accumulation in the light. HMR binds more strongly to the active form of phytochromes. This interaction is mediated by the photosensory domains of phytochromes and two phytochrome-interacting regions in HMR. Missense mutations in either HMR or phyB that alter the phytochrome/HMR interaction can also change HMR levels and photomorphogenetic responses. HMR accumulation in a constitutively active *phyB* mutant (*YHB*) is required for *YHB*-dependent PIF3 degradation in the dark. Our genetic and biochemical studies strongly support a novel phytochrome signaling mechanism in which photoactivated phytochromes directly interact with HMR and promote HMR accumulation, which in turn mediates the formation of photobodies and the degradation of PIFs to establish photomorphogenesis.

[**Keywords:** phytochrome; photomorphogenesis; photobody; HEMERA; phytochrome-interacting factors]

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Light plays an important role in almost every facet of plant growth and development, from seed germination to floral initiation (Franklin and Quail 2010; Kami et al. 2010). The profound impact of light on plant development is best exemplified during seedling establishment, where the absence or presence of light leads to morphologically distinct developmental programs. *Arabidopsis* seedlings grown in the dark adopt a developmental program called skotomorphogenesis, which inhibits leaf development in order to allocate energy to elongating the embryonic stem, called the hypocotyl. Hypocotyl elongation enables seedlings to emerge from the soil after the seed germinates in the ground. In contrast, when *Arabidopsis* seedlings are exposed to light, they initiate a morphologically distinct program called photomorphogenesis, which restricts hypocotyl elongation and establishes a body plan for a photoautotrophic lifestyle, including

leaf expansion, chloroplast differentiation, and photosynthesis (Chen and Chory 2011). Photomorphogenetic responses are driven by complex cascades of transcriptional regulation and massive reprogramming of the *Arabidopsis* genome (Ma et al. 2001; Tepperman et al. 2006; Hu et al. 2009; Leivar et al. 2009); up to one-third of *Arabidopsis* genes are differentially expressed between skotomorphogenesis and photomorphogenesis (Ma et al. 2001). This developmental transition is orchestrated by sophisticated light signaling pathways that are vital for the survival of young seedlings.

Photomorphogenesis is initiated by a suite of photoreceptors that are capable of sensing distinct colors of light (Kami et al. 2010). Among these photoreceptors, the red (R) and far-red (FR) light-sensing phytochromes (phys) are essential to establish photomorphogenesis (Franklin and Quail 2010). Phys are bilin-containing proteins that can be photoconverted between two relatively stable forms: a red light-absorbing inactive Pr form and a far-red light-absorbing active Pfr form (Rockwell et al. 2006; Nagatani 2010). In *Arabidopsis*, phyA and phyB are the most

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prominent phys, and they monitor continuous FR and R light, respectively. Whereas *phy* mutants are blind to R and FR light and consequently display elongated hypocotyls under these conditions (Nagatani et al. 1993; Reed et al. 1993; Strasser et al. 2010), the constitutively active *phyB* mutant *YHB*, which contains a Y276H mutation, is able to turn on photomorphogenesis in the dark (Su and Lagarias 2007).

A central mechanism by which phys rapidly regulate gene expression is to induce the degradation of a group of phy-interacting transcription factors called PIFs (Leivar and Quail 2011). The PIFs are a group of basic helix–loop–helix (bHLH) transcription factors that interact directly and preferentially with the Pfr form of *phyA*, *phyB*, or both (Leivar and Quail 2011). PIF3, the founding member of the PIF family, was identified by Quail's laboratory (Ni et al. 1998) through a yeast two-hybrid screen using *phyB* as the bait. Since then, six additional PIFs and PIF3-like (PIL) transcription factors have been identified: PIF1/PIL5 (Huq et al. 2004; Oh et al. 2004), PIF4 (Huq and Quail 2002), PIF5/PIL6 (Makino et al. 2002; Khanna et al. 2004), PIF6/PIL2 (Salter et al. 2003; Khanna et al. 2004), PIF7 (Leivar et al. 2008a), and PIF8 (Leivar and Quail 2011). PIF1, PIF3, PIF4, PIF5, and PIF7 have been found to bind specifically to the G-box motif (CACGTG) and regulate the expression of thousands of light-responsive genes (Martinez-Garcia et al. 2000; Huq and Quail 2002; Oh et al. 2007, 2009; de Lucas et al. 2008; Feng et al. 2008; Leivar et al. 2008a; Moon et al. 2008; Hornitschek et al. 2009; Toledo-Ortiz et al. 2010; Cheminant et al. 2011; Franklin et al. 2011; Kunihiro et al. 2011; Li et al. 2012; Soy et al. 2012). Most PIFs accumulate to high levels in the dark; they are essential for skotomorphogenesis and act as antagonistic factors for phy-mediated responses (Leivar and Quail 2011). For example, PIF3 promotes hypocotyl elongation and represses chloroplast differentiation (Kim et al. 2003; Stephenson et al. 2009; Soy et al. 2012). Photoactivation of phys triggers the phosphorylation and rapid degradation of most PIFs to turn on photomorphogenesis (Bauer et al. 2004; Shen et al. 2005, 2007; Al-Sady et al. 2006; Oh et al. 2006; Nozue et al. 2007; Lorrain et al. 2008). Consistent with this model, knocking out four PIF genes in the *pif1/pif3/pif4/pif5* quadruple mutant (*pifq*) is sufficient to turn on photomorphogenesis in the dark, similar to the *YHB* allele of *phyB* (Leivar et al. 2008b, 2009; Shin et al. 2009). The extensive and elegant studies performed on PIFs in the past decade have established a link between phy photoactivation, gene regulation, and downstream light responses. However, the upstream signaling mechanisms mediating PIF degradation are still largely unknown.

Accumulating evidence links the degradation of PIFs to their subnuclear compartmentalization in phy-containing subnuclear domains called photobodies (Bauer et al. 2004; Al-Sady et al. 2006; Van Buskirk et al. 2012). At the cellular level, the translocation of phys from the cytoplasm to photobodies is one of the earliest light responses (Yamaguchi et al. 1999; Kircher et al. 2002; Bauer et al. 2004; Chen and Chory 2011). The localization of *phyB* to a few large photobodies (1–2 μ m in diameter) is directly

regulated by light quality and quantity and is closely correlated with phy-mediated responses, such as hypocotyl growth inhibition (Yamaguchi et al. 1999; Kircher et al. 2002; Chen et al. 2003; Su and Lagarias 2007; M Chen et al. 2010). During the dark-to-light transition, PIF3 colocalizes with *phyA* and *phyB* to photobodies before its degradation (Bauer et al. 2004; Al-Sady et al. 2006). The recruitment of PIF3 to photobodies depends on the direct interaction between PIF3 and phys (Al-Sady et al. 2006; Oka et al. 2008; Kikis et al. 2009). Mutations in the *phyA*- and *phyB*-binding domains of PIF3 block both PIF3 localization to photobodies and its degradation during the dark-to-light transition (Al-Sady et al. 2006). These results led to the hypothesis that photobodies might be sites for PIF3 degradation. Consistent with this model, using a *phyB*-GFP mislocalization screen, we recently identified a novel phy-specific signaling component called HEMERA (HMR) (M Chen et al. 2010). In *hmr* mutants, *phyB*-GFP fails to localize to large photobodies; as a result, the degradation of PIF1 and PIF3 is impaired in the light (M Chen et al. 2010). In addition, HMR is structurally similar to the yeast multiubiquitin-binding protein Rad23, and HMR may therefore be directly involved in protein degradation. Moreover, in the nucleus, HMR localizes to the periphery of photobodies, further supporting the role of photobodies in PIF degradation (M Chen et al. 2010).

Although genetic evidence strongly suggests that HMR is a key phy signaling component that is essential for the photobody localization of *phyB*-GFP and the proteolysis of PIF1 and PIF3, the molecular mechanism linking phys and HMR is completely unknown. In this study, we uncover a novel signaling mechanism in which photoactivated phys directly bind to HMR and promote HMR accumulation as well as the subsequent degradation of PIFs to establish photomorphogenesis.

Results

Phys promote HMR accumulation in the light

We previously reported that HMR accumulates to higher levels in the light than in the dark (M Chen et al. 2010). Therefore, we further examined HMR protein accumulation in monochromatic R and FR light and in the true-dark condition, in which *phyB* molecules in the Pfr form are inactivated by a FR pulse after seed germination (Leivar et al. 2009). As shown in Figure 1A, the steady-state level of HMR was threefold to fourfold higher in either R or FR light compared with the true-dark condition. Because the transcript level of *HMR* was not significantly changed under these conditions (Fig. 1B), it suggests that HMR is regulated post-transcriptionally. Consistent with this notion, when HA-tagged HMR was expressed under the 35S constitutive promoter in a *hmr*-null allele background (*hmr-5*, described below), the *HMR::HA* transcript level was 23-fold higher than that of the endogenous *HMR* level in wild-type Col-0 seedlings. However, the protein level of *HMR::HA* was similar to that of the endogenous HMR in Col-0 (Fig. 1C,D).

Figure 1. Light promotes HMR accumulation in a phy-dependent manner. (A) Western blot analysis of HMR protein levels in 4-d-old Col-0 seedlings grown in true-dark, $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ of R light, or $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ of FR light. RPN6 was used as a loading control. (B) Quantitative RT-PCR analysis of the steady-state *HMR* mRNA levels in Col-0 seedlings grown in the same conditions as described in A. (C) Quantitative RT-PCR analysis of *HMR* or *HMR::HA* mRNA levels in 4-d-old Col-0 and *HMR::HA/hmr-5* seedlings grown in $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ of R light. The steady-state mRNA level of *HMR::HA* in *HMR::HA/hmr-5* was 23-fold higher than that of *HMR* in Col-0. (D) Western blot analysis of HMR or *HMR::HA* protein levels in Col-0 and *HMR::HA/hmr-5* seedlings grown in the same conditions as described in C. (E) Western blot analysis of HMR protein levels in 4-d-old Col-0 seedlings grown in

true-dark or pseudo-dark conditions. (F) Western blot analysis of HMR protein levels in 4-d-old *phyA-211* and *phyB-9* seedlings grown in the conditions described in A. (G) Western blot analysis of total and nuclear HMR levels in 4-d-old Col-0 seedlings grown under the conditions described in A. RNA polymerase II and ferredoxin-sulfite reductase (SiR) were used as markers for the nuclear and plastidic fractions, respectively. The relative HMR levels, normalized to RPN6, are shown below the HMR blots in A, E, and F. The asterisks indicate nonspecific bands. In B and C, transcript levels were calculated relative to those of *PP2A*. Error bars indicate the standard deviation of three replicate reactions.

Because the *HMR::HA* construct was able to fully complement *hmr-5* (Supplemental Fig. S1), the *HMR::HA* protein should be functional and presumably behaves similarly to HMR. These results demonstrate that the amount of HMR does not correlate with the *HMR* transcript level and is tightly controlled to within a narrow range.

To determine how sensitive HMR accumulation is to light, we examined HMR levels in Col-0 under a range of R light intensities and found that the HMR level reached a plateau under very dim ($0.2 \mu\text{mol m}^{-2} \text{s}^{-1}$) R or FR light (Supplemental Fig. S2). More interestingly, when comparing Col-0 seedlings grown under true-dark with those under pseudo-dark, where a residual amount of phyB protein remains in the active Pfr form (Leivar et al. 2008b), HMR accumulated twofold more in the pseudo-dark condition (Fig. 1E), suggesting that HMR accumulation is extremely sensitive to light and/or to the Pfr form of phys. In contrast to the high sensitivity of the steady-state level of HMR to light, the kinetics of HMR accumulation were rather slow during the dark-to-light transition: It took ~ 24 h for HMR to reach a plateau during the dark-to-R light transition (Supplemental Fig. S3). This might suggest that HMR accumulation requires prolonged light exposure.

We then asked whether HMR accumulation is dependent on phys. We carefully examined HMR levels in *phyA* and *phyB* mutants under dark, R, and FR conditions. In the *phyA-211* mutant, HMR accumulation was intact in R light but was almost completely abolished in FR light (Fig. 1F), suggesting that HMR accumulation in FR is *phyA*-dependent. In contrast, HMR levels were only twofold higher in R light-grown *phyB-9* mutants compared with dark-grown *phyB-9* mutants (Fig. 1F). This result suggests that *phyB* plays a prominent role in HMR accumulation in R light but that other phys, such as *phyC-E*, may play a role as well. Consistent with these

results, the accumulation of *HMR::HA* was also phy-dependent in the corresponding transgenic lines (Supplemental Fig. S4). Taking these results together, we conclude that photoactivated phys promote HMR accumulation in the light.

It has been previously shown that HMR protein is localized to both the nucleus and chloroplasts (M Chen et al. 2010). To examine whether phys can directly affect the nuclear pool of HMR, we analyzed HMR levels from the nuclear fractions of dark- and light-grown seedlings. Indeed, the nuclear pool of HMR was increased in the light (Fig. 1G), suggesting that the increase in total HMR is due at least in part to the increase in nuclear HMR.

HMR preferentially binds to the active Pfr form of phys

Molecular and genetic characterization of the *hmr* mutant has shown that HMR is required for multiple early phy signaling events, suggesting that HMR acts early in phy signaling (M Chen et al. 2010). Therefore, we hypothesized that one possible mechanism by which phys promote HMR accumulation could be through a direct protein-protein interaction. To test this hypothesis, we examined whether glutathione S-transferase (GST)-tagged HMR (GST-HMR) could pull down in vitro translated *phyA* and *phyB*. Indeed, GST-HMR was able to pull down both the *phyA* and *phyB* apoproteins (Fig. 2A). To test whether this interaction is dependent on light, we reconstituted the holoproteins of *phyA* and *phyB* by incorporating a phycocyanobilin chromophore isolated from the blue-green algae *Spirulina platensis* (Terry et al. 1993) and then performed the GST pull-down assays under either R or FR light. These experiments showed that the interaction between HMR and phys is light-dependent, as GST-HMR interacted more strongly with the active Pfr forms of both *phyA* and *phyB* (Fig. 2A).

Figure 2. Phys directly interact with HMR in a light-dependent manner. (A) Pull-down assays using either GST or GST-HMR proteins to pull down in vitro translated phyA or phyB. The pull-down assays were carried out with the phy apoprotein (apo) or the holoprotein in either the Pfr or Pr form as indicated. (Top) Bound and input phy fractions were detected via Western blot using anti-phyA or anti-phyB antibodies. Immobilized GST and GST-HMR (arrows) are shown in the Coomassie Blue-stained gel (CB). The relative amounts of the bound phy proteins, normalized to the amount of the corresponding GST-HMR bait (CB), are shown below the bound phy blots. (B) Coimmunoprecipitation of HMR::HA and phyA. Coimmunoprecipitation experiments were carried out with protein extract from 4-d-old *HMR::HA/hmr-5* and *Col-0* seedlings grown in $0.5 \mu\text{mol m}^{-2} \text{s}^{-1}$ of FR light. HMR::HA was immunoprecipitated using an anti-HA-coupled matrix. The input and the immunoprecipitated HMR::HA and phyA were detected by Western blot (WB) with anti-HA and anti-phyA antibodies as indicated.

To confirm the phy/HMR interaction in vivo, we performed coimmunoprecipitation experiments using the *HMR::HA* transgenic line. It was previously reported that R light induces the pelletability of phys (Quail and Briggs 1978). Consistent with this notion, we found that phyB precipitated in plant protein extracts under R light (data not shown), which made it difficult to coimmunoprecipitate phyB and HMR under R light. Therefore, we focused on the phyA/HMR interaction under FR light. As expected, endogenous phyA could be coimmunoprecipitated with HMR::HA using anti-HA antibodies (Fig. 2B), suggesting that phyA interacts with HMR in vivo. These results provide the initial evidence supporting a model in which the photoactivation of phys promotes HMR accumulation through a direct interaction.

Domains required for the interaction between phys and HMR

The domain structure of phys is well-defined. In higher plants, phys consist of a photosensory N-terminal half for signaling and a C-terminal half for dimerization and nuclear localization (Rockwell et al. 2006; Nagatani 2010). To determine the phy domains involved in the

interaction with HMR, we used phyA as a model and generated phyA N-terminal (phyA-N) and C-terminal (phyA-C) truncation fragments by in vitro transcription/translation (Fig. 3A). GST-HMR was able to pull down only phyA-N but not phyA-C (Fig. 3B), suggesting that HMR specifically interacts with the N-terminal half of phyA. The N-terminal half of phys includes the NTE (N-terminal extension), the PAS (PER, ARNT, and SIM) domain, the GAF (cGMP phosphodiesterase, adenylate cyclase, FhlA) domain, and the PHY (phytochrome-specific GAF-related) domain. Both the GAF and PHY domains are important for photosensing: The GAF domain contains the chromophore attachment site and is essential for photosensing, and the PHY domain is required to stabilize the Pfr form of phy (Oka et al. 2004). Because the phy/HMR interaction is light-dependent (Fig. 2A,B), we generated a phyA fragment containing only the GAF and PHY domains (phyA-GP) and asked whether HMR could directly interact with these photosensory domains. Indeed, GST-HMR could pull down phyA-GP (Fig. 3A,B), suggesting that the photosensory GAF/PHY domains in phyA are sufficient to bind HMR. Because the GAF/PHY domains are highly conserved between phyA and phyB, it is likely that HMR interacts with the same domains in phyB.

The primary amino acid sequence of HMR does not reveal any known functional domains. To dissect the domains in HMR that are required for the phy interaction, we fused GST to various HMR truncations and tested which fragment was able to pull down in vitro translated phyA-N. These experiments show that the fragment containing amino acids 1–352 (GST-HMRn) was both required and sufficient for phyA binding (Fig. 3C,D). The N terminus of HMR was then further dissected into three fragments, and two phyA-interacting regions (PIRs) were discovered: PIR1 (amino acids 1–115) and PIR2 (amino acids 252–352). PhyA interacted with PIR1 considerably more strongly than with PIR2.

The interaction between phys and HMR is required for HMR accumulation

To demonstrate the significance of the phy/HMR interaction in promoting HMR accumulation, we searched for HMR missense mutations that might result in defective phy interactions and then asked whether these mutations could also attenuate HMR accumulation. Using the *Arabidopsis* TILLING service (<http://tilling.fhcr.org>), we identified 10 new *hmr* alleles, *hmr-4* to *hmr-13*, with mutations spanning from the beginning of HMR to the end of PIR2 (Supplemental Fig. S5A; Supplemental Table S1). Among the new alleles, only *hmr-5* and *hmr-12* showed long hypocotyl phenotypes in R and FR light and albino phenotypes in R light (Fig. 4A,B; Supplemental Fig. S5B,C). While *hmr-5* contains a premature stop codon, *hmr-12* is a missense allele that replaces Arg 293 with a Lys residue (Supplemental Fig. S5A). Interestingly, Arg 293 lies in the middle of PIR2 and is highly conserved among all sequenced HMR orthologs from moss to higher plants (Fig. 4C). These observations

Figure 3. The interaction between phys and HMR requires the photosensory domains of phys and two phy-interacting regions in HMR. (A) Schematic illustration of the bait (GST-HMR) and the prey fragments (phyA apoproteins, including phyA-N, phyA-C, and phyA-GP) used in the GST pull-down assays shown in **B**. (B) Pull-down assays using GST and GST-HMR to pull down phyA apoprotein fragments. (Top) Bound and input fractions of the phyA fragments were detected by Western blot using anti-HA antibodies. Immobilized GST and GST-HMR (arrows) are shown in the Coomassie Blue-stained gel (CB). (C) Schematic illustration of GST-fused HMR truncation fragments, including GST-HMRn, GST-HMRc, GST-PIR1, GST-GN (Glu/NLS), and GST-PIR2 used as the baits and the phyA-N apoprotein fragment used as the prey in the pull-down assays in **D**. (D) Pull-down assays using GST, GST-HMR, and GST-fused HMR truncations to pull down phyA-N. (Top) Bound and input phyA-N fractions were detected by Western blot using anti-HA antibodies. Immobilized GST and GST-HMR are shown in the Coomassie Blue-stained gel (CB).

prompted us to test whether the R293K mutation had any effect on the phy/HMR interaction. First, we examined whether full-length HMR^{R293K} could pull down phyA using in vitro GST pull-down assays. Unexpectedly, full-length HMR^{R293K} was able to pull down phyA in a manner similar to wild-type HMR (Supplemental Fig. S6). We reasoned that HMR^{R293K} might only slightly alter the phyA/HMR interaction and that this small defect in the phyA/PIR2 interaction could be masked by the strong interaction between phyA and PIR1 (Fig. 3D). To investigate this possibility, we compared the ability of GST-PIR2 and GST-PIR2^{R293K} to bind phyA. Indeed, the R293K mutation significantly weakened the interactions between PIR2 and both phyA and phyB (Fig. 4D). We then asked whether the R293K mutation had any effect on HMR^{R293K} accumulation. To our surprise, despite the small defect in phy interaction in vitro, HMR^{R293K} accumulation was dramatically reduced in *hmr-12* seedlings grown under either R or FR light (Fig. 4E). These results provide genetic evidence supporting the model that HMR accumulation in the light is dependent on its direct interaction with phys.

YHB interacts more strongly with HMR and promotes HMR accumulation in the dark

To further test the model that HMR accumulation is dependent on its interaction with phys, we asked whether the constitutively active phyB mutant YHB could enhance the interaction between phy and HMR and consequently enhance HMR accumulation. The YHB mutant contains a Y276H mutation that locks phyB in its active form; as a result, it can trigger photomorphogenesis in the

dark (Su and Lagarias 2007; Hu et al. 2009). Interestingly, the interaction between GST-HMR and YHB is not light-dependent (Fig. 5A). Consistent with the notion that HMR interacts more strongly with the active form of phys, GST-HMR interacted more strongly with YHB than with wild-type phyB under both R and FR light (Fig. 5A). This difference was more significant under FR light, where phyB was in the inactive form and YHB remained in the active form (Fig. 5A). We then tested whether YHB could promote HMR accumulation in the dark. If HMR accumulation is dependent on the phyB/HMR interaction, HMR should accumulate to higher levels in YHB seedlings. Thus, we compared HMR levels between dark-grown YHB seedlings and PBG seedlings, which overexpress the wild-type phyB (Yamaguchi et al. 1999). Consistent with our hypothesis, dark-grown YHB seedlings accumulated dramatically more HMR than dark-grown PBG seedlings (Fig. 5C). Moreover, similar to Col in the light, the nuclear pool of HMR was also increased in YHB compared with PBG (Supplemental Fig. S7). These results further demonstrate that the accumulation of HMR generally, and the nuclear pool of HMR specifically, are dependent on its interaction with phys.

To further test this model, we asked whether HMR^{R293K} could also interact more strongly with YHB and whether the defect in HMR^{R293K} accumulation in *hmr-12* could be rescued by YHB. In vitro pull-down assays showed that GST-PIR2^{R293K} behaved similarly to GST-PIR2 and interacted more strongly with YHB than with phyB (Supplemental Fig. S8A). In addition, HMR^{R293K} accumulated to a higher level in the *hmr-12*/YHB double mutant than in *hmr-12* in continuous R light

Figure 4. The R293K mutation in the PIR2 of HMR reduces the phy/HMR interaction and abolishes HMR accumulation in the light. (A) Images of 4-d-old Col-0, *hmr-2*, *hmr-5*, and *hmr-12* seedlings grown in either 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$ R light or 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$ FR light. Bar, 3 mm. (B) R and FR light fluence response curves for Col-0, *hmr-2*, *hmr-5*, and *hmr-12*. The Y-axis represents hypocotyl length relative to the dark-grown hypocotyl length of each respective genotype. Error bars indicate standard deviation. (C) Sequence alignment of the portion of PIR2 containing R293 from HMR orthologs. Identical and similar amino acids are shaded in black and gray, respectively. Arg 293 in *Arabidopsis* HMR, highlighted with a red box, is conserved in all HMR orthologs from *Physcomitrella patens* to higher plants. Accession numbers of the protein

sequences used in the alignment are listed in Supplemental Table S2. (D) Pull-down assays using GST-PIR2 and GST-PIR2^{R293K} to pull down phyA (left) and phyB (right) holoproteins under R light. phyA and phyB were detected by Western blots using anti-phyA and anti-phyB antibodies. Immobilized GST-PIR2 and GST-PIR2^{R293K} are shown in the Coomassie Blue-stained gel (CB). The relative amounts of the bound phy proteins were normalized to the amount of the corresponding GST fusion protein bait (CB) and are shown below the bound phy blots. (E) Western blot analysis of HMR protein levels in 4-d-old Col-0, *hmr-2*, *hmr-5*, and *hmr-12* seedlings grown in 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of R light (top) or 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of FR light (bottom). HMR^{R293K} in *hmr-12* failed to accumulate in the light. RPN6 was used as a loading control.

(Supplemental Fig. S8B), suggesting that YHB can enhance HMR^{R293K} accumulation, most likely due to a stronger interaction between HMR^{R293K} and YHB. More interestingly, the tall hypocotyl phenotype of *hmr-12* was partially rescued in the *hmr-12/YHB* double mutant (Supplemental Fig. S8C,D). Because *YHB* failed to rescue the null *hmr-1* allele in the *hmr-1/YHB* double mutant (Supplemental Fig. S8C,D), it is most likely that *YHB* rescues *hmr-12* by promoting the accumulation of HMR^{R293K}. The fact that HMR^{R293K} accumulation could be enhanced by *YHB* further supports the notion that the HMR^{R293K} accumulation defect is due to its weaker interaction with phys, rather than a general structural defect caused by the R293K mutation. Taken together, these results further support the conclusion that HMR accumulation depends on its interaction with activated phys.

HMR accumulation is required for phy signaling and PIF degradation

A key mechanism by which phys turn on photomorphogenesis is to remove the antagonistically acting PIFs (Leivar and Quail 2011). Because HMR has been shown to be essential for the formation of large phyB-containing photobodies and for the degradation of PIF1 and PIF3 in the light, it was proposed that HMR may mediate phy signaling through the regulation of PIFs (M Chen et al. 2010). Because *YHB* mutants accumulate HMR in the dark (Fig. 5C), the *YHB* mutant allowed us to further test whether PIF degradation is dependent on HMR accumulation in *YHB*. We previously showed that the *hmr-1/YHB* double mutant partially reverses the short hypocotyl phenotype of *YHB* in the dark, which suggests that

HMR accumulation is required for YHB signaling (Fig. 5B; M Chen et al. 2010). Because hypocotyl elongation in the dark is promoted by at least four PIFs, including PIF1, PIF3, PIF4, and PIF5 (Leivar et al. 2008b; Shin et al. 2009), we first asked whether the photomorphogenetic phenotypes of *YHB* in the dark could be due to the removal of PIFs. Using PIF3 as a model, we compared PIF3 protein levels in dark-grown *YHB*, dark-grown PBG, and R light-grown PBG seedlings. As expected, PIF3 accumulated in the dark-grown PBG seedlings and disappeared in both the dark-grown *YHB* and light-grown PBG seedlings (Fig. 5C). This result is consistent with the model that photoactivation

Figure 5. YHB strongly interacts with HMR and promotes HMR accumulation in the dark, and HMR accumulation is required for YHB-triggered PIF3 degradation. (A) Pull-down assays using GST-HMR to pull down phyB or YHB in either R or FR light. Immobilized GST-HMR is shown in the Coomassie Blue-stained gel (CB). The relative amounts of the bound phy proteins, normalized to the amount of the corresponding GST-HMR bait (CB), are shown below the bound phy blots. (B) Images of 4-d-old *YHB*, *hmr-1/YHB*, and PBG seedlings grown in true-dark and PBG grown in 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of R light. Bar, 3 mm. (C) Western blot analysis of HMR and PIF3 protein levels in the seedlings shown in B. RPN6 was used as a loading control.

of phyB leads to PIF degradation (Al-Sady et al. 2006); YHB is able to trigger PIF3 degradation in the dark. We then asked whether the disappearance of PIF3 in dark-grown YHB could be reversed in the absence of HMR. Indeed, the PIF3 accumulation defect in YHB was reversed in the *hmr-1/YHB* double mutant: *hmr-1/YHB* seedlings in the dark accumulated PIF3 to levels comparable with those of dark-grown PBG seedlings (Fig. 5C). Because the transcript level of *PIF3* was only increased less than twofold in *hmr-1/YHB* compared with YHB in the dark (Supplemental Fig. S9A), the accumulation of PIF3 in *hmr-1/YHB* is more likely due to the stabilization of PIF3 in the absence of HMR. These results further demonstrate that photoactivation of phys is not sufficient for PIF3 degradation and that the removal of PIF3 is HMR-dependent.

Because phyA degradation is also dependent on light and HMR, we asked whether the amount of phyA behaved similarly to PIF3. However, as shown in Supplemental Figure S9, B and C, despite alterations in the *PHYA* transcript levels, the protein levels of phyA in dark-grown YHB and *hmr-1/YHB* lines were the same as those of dark-grown PBG seedlings. Therefore, the long hypocotyl phenotype of the *hmr-1/YHB* double mutant is independent of phyA and can be explained at least in part by PIF3 accumulation.

To further demonstrate that HMR transduces phy signals through PIFs, we examined the expression of PIF target genes in the YHB and *hmr-1/YHB* backgrounds. The YHB- and PIF-dependent genes are well characterized (Hu et al. 2009; Leivar et al. 2009; Shin et al. 2009). In addition, a group of PIF direct target genes has been identified. We picked 12 well-characterized PIF-dependent genes, including six PIF-induced genes and six PIF-repressed

genes. Among them, *GUN5*, *SIGE*, *PIL1*, *ATHB2*, *XTR7*, and *HFR1* have been shown to be the direct targets of PIFs (Monte et al. 2004; de Lucas et al. 2008; Feng et al. 2008; Hornitschek et al. 2009; Cheminant et al. 2011; Kunihiro et al. 2011). To test whether HMR is involved in the degradation of not only PIF1 and PIF3, but also PIF4 and PIF5, we included not only PIF1- and PIF3-dependent genes, such as *SIGE* and *GUN5*, but also a number of PIF4 and PIF5 target genes, including *PIL1*, *XTR7*, *HFR1*, *ATHB2*, and *LHCB2.4*. Consistent with the fact that both dark-grown YHB and light-grown PBG lines lack PIF3 accumulation, they also shared similar expression patterns for all 12 genes (Fig. 6A,B). In contrast, dark-grown PBG seedlings showed the opposite expression patterns compared with dark-grown YHB and light-grown PBG seedlings, as PIF3 accumulated in dark-grown PBG. In dark-grown *hmr-1/YHB* double mutant seedlings compared with YHB, the six PIF-repressed genes were down-regulated, and the six PIF-induced genes were up-regulated (Fig. 6A,B). These results support a model in which the tall hypocotyl phenotype of the *hmr-1/YHB* double mutant is due to the alteration of both PIF levels and the expression of PIF-regulated genes. Because the expression of all 12 PIF target genes, including the PIF4 and PIF5 targets, was partially reversed in the *hmr-1/YHB* mutants compared with YHB, it implies that HMR regulates not only PIF1 and PIF3, but also PIF4 and PIF5 as well as the expression of their target genes.

Discussion

Early phy signaling events include the translocation of phys from the cytoplasm to photobodies as well as the

Figure 6. HMR accumulation is required for the regulation of PIF target genes. Quantitative RT-PCR analysis of PIF-repressed (A) and PIF-induced (B) genes in 4-d-old YHB, *hmr-1/YHB*, and PBG seedlings grown in the indicated conditions. Transcript levels were calculated relative to *PP2A*. The fold decrease (A) or increase (B) of the expression levels between dark-grown YHB and dark-grown *hmr-1/YHB* is shown in each panel. Error bars indicate the standard deviation of three replicates.

rapid degradation of PIFs (Chen and Chory 2011; Leivar and Quail 2011). HMR was identified as a phy signaling component required for both of these early events (M Chen et al. 2010). Here, we provide evidence supporting a novel light signaling mechanism by which photoactivated phys directly interact with HMR and promote HMR accumulation in the light, and HMR accumulation facilitates photobody localization of phys and subsequent PIF degradation to establish photomorphogenesis (Fig. 7).

The first light response mediated by phys at the molecular level is the photoconversion of phy from the Pr form to the Pfr form. The conformational changes accompanied by the photoactivation of phys alter the binding affinities between phy and phy-interacting proteins, and these structural and binding property changes mediate early phy signaling events (Rockwell et al. 2006; Bae and Choi 2008; Nagatani 2010). A number of phy-interacting proteins have been identified that bind preferentially to the Pfr form of phys. One example is *FHY1* (far-red elongated hypocotyl 1) and its homolog, *FHL* (*FHY1*-like). *FHY1* and *FHL* bind to the Pfr form of phyA and mediate both phyA nuclear accumulation and signaling (Hiltbrunner et al. 2005, 2006; Genoud et al. 2008; Shen et al. 2009; Yang et al. 2009). Another well-studied example is the family of PIF proteins, which have been suggested to regulate phyB protein levels and mediate the expression of hundreds of light-responsive genes (Al-Sady et al. 2008; Jang et al. 2010; Leivar and Quail 2011). Here, we show that HMR is another direct binding partner of both phyA and phyB. The phy/HMR interaction is light-regulated, as GST-HMR interacts preferentially with the Pfr form of both phyA and phyB as well as with the constitutively active YHB mutant (Figs. 2A, 5A). This light-dependent phy/HMR interaction could be explained by the fact that HMR interacts with the photosensory GAF/PHY domains of phyA and likely phyB as well (Fig. 3). Therefore, conformational changes between the Pr and Pfr forms of phys could have a direct impact on HMR binding. We further confirmed the phy/HMR interaction in vivo by coimmunoprecipitation of HMR-HA and phyA (Fig. 2C). The direct interactions between HMR and both phyA and phyB suggest that HMR participates in the early signaling steps downstream from both phyA and phyB. Consistent with this conclusion, previous and

current genetic studies show that HMR acts downstream from phyA and phyB and is required for multiple early phy signaling events as well as for YHB signaling in the dark (Fig. 5; M Chen et al. 2010). Although we mainly focused on the light-dependent interaction between HMR and phy holoproteins, we also consistently observed that GST-HMR interacted more strongly with the phy apoproteins than with the corresponding holoproteins (Fig. 2A). This could be an artifact due to contaminants from the phycocyanobilin extract. However, we cannot exclude the possibility that there is biological significance for the strong interaction between HMR and phy apoproteins. For example, HMR could bind to phy apoproteins and facilitate their chromophorylation. However, our previous result showing YHB fluorescence in the *hmr-1* background argues against this hypothesis (M Chen et al. 2010).

The phy/HMR interaction is required for phy signaling, and this conclusion is supported by several lines of evidence. First, the phy/HMR interaction is required for the accumulation of HMR in the light. Supporting the cause/effect relationship between this interaction and HMR accumulation, single amino acid substitutions in either HMR (*HMR*^{R293K} in *hmr-12*) or phyB (YHB) altered the affinity between phys and HMR and also dramatically affected HMR accumulation (Figs. 4, 5). In addition, the nuclear pool of HMR was increased in both light-grown Col-0 and dark-grown YHB seedlings relative to the dark-grown controls (Fig. 1G; Supplemental Fig. S7), suggesting that the accumulation of HMR in the nucleus might be a direct consequence of the phy/HMR interaction. Second, the phy/HMR interaction may be important in regulating phy localization to photobodies, as HMR is essential for at least phyB to localize to large photobodies. Although the localization of phyA in the *hmr* mutant has not been investigated, phyA degradation is impaired in *hmr* mutants (M Chen et al. 2010), which implies that phyA localization to photobodies could also be regulated by HMR.

Light-triggered PIF degradation is a central mechanism to switch from skotomorphogenesis to photomorphogenesis (Leivar et al. 2008b; Shin et al. 2009). The current model suggests that photoactivated phys interact with PIFs and trigger their phosphorylation as well as their subsequent degradation (Bauer et al. 2004; Shen et al. 2005, 2007; Al-Sady et al. 2006; Oh et al. 2006; Nozue et al. 2007; Lorrain et al. 2008). However, the mechanism of PIF degradation is unknown (Leivar and Quail 2011). Because PIF3 localizes to photobodies with phyA and phyB prior to its degradation, it was proposed that PIFs could be recruited to photobodies for degradation (Bauer et al. 2004; Al-Sady et al. 2006). This hypothesis has been supported by several lines of evidence. First, mutations in the phyA- and phyB-binding domains of PIF3 block PIF3 localization to photobodies as well as PIF3 degradation during the dark-to-light transition (Al-Sady et al. 2006), suggesting that the recruitment of PIF3 to photobodies is phy-dependent. Second, mutations in the light-sensing knot of phyB disrupt its interaction with multiple PIFs without affecting phyB's localization to photobodies.

Figure 7. Model for early phy signaling events. Photoactivated phys directly bind to HMR and promote HMR accumulation, which in turn facilitates photobody formation and PIF degradation on photobodies; in parallel, phys interact with PIFs to potentiate their degradation by triggering PIF phosphorylation.

However, these phyB mutants are not functional (Oka et al. 2008; Kikis et al. 2009). These results suggest that the localization of phyB to photobodies does not require the interaction with PIFs and that the recruitment of PIFs to photobodies by phys is required for phy signaling. Third, the role of photobodies in PIF degradation is further supported by the characterization of the *hmr* mutant, in which phyB-GFP fails to localize to large photobodies. Consequently, both PIF1 and PIF3 fail to be degraded in the light (M Chen et al. 2010). It was also shown that HMR is structurally similar to the yeast multiubiquitin-binding protein Rad23 and that it is localized to the periphery of photobodies, which raises the possibility that HMR itself is directly involved in PIF degradation (M Chen et al. 2010). In this study, we further substantiate this model by characterizing PIF3 levels in the *YHB* and *hmr-1/YHB* double mutants. We showed that the *YHB* mutant is able to trigger PIF3 degradation in the dark (Fig. 5C), suggesting that the constitutively photomorphogenetic phenotype of *YHB* is at least in part due to the removal of PIFs. More interestingly, we showed that *YHB* is not sufficient for PIF degradation, as *hmr-1/YHB* double mutants partially reversed the PIF3 degradation phenotype of *YHB* (Fig. 5C). This result suggests that the presence of HMR is essential for PIF degradation. We previously reported that HMR is required for *YHB* to localize to large photobodies in the dark (M Chen et al. 2010). Therefore, our present study, combined with our previous results, supports a model in which HMR accumulation facilitates photobody localization of photoactivated phyB and the subsequent degradation of PIFs (Fig. 7). Although this model suggests that PIFs are degraded on photobodies, there is still a possibility that PIFs are only modified on photobodies and are degraded elsewhere (Van Buskirk et al. 2012). Additional evidence is still needed to discern these possibilities.

The establishment of photomorphogenesis by phys in the light is mediated by not only the removal of the negatively-acting PIFs, but also the accumulation or stabilization of a number of positively acting factors. These factors include HY5 (elongated hypocotyl 5) (Koornneef et al. 1980; Oyama et al. 1997), LAF1 (long after far-red light 1) (Ballesteros et al. 2001), HFR1 (long hypocotyl in far-red 1) (Fairchild et al. 2000), and some members of the B-box zinc finger family (BBX) (Kumagai et al. 2008; Khanna et al. 2009), such as CONSTANS (CO)/BBX1 (Laubinger et al. 2006; Liu et al. 2008), COL3 (CONSTANS-like 3)/BBX4 (Datta et al. 2006), LZFI (light-regulated zinc finger protein 1)/STH3 (salt tolerance homolog 3)/BBX22 (Chang et al. 2008, 2011; Datta et al. 2008), and BBX21/STH2 (salt tolerance homolog 2) (Datta et al. 2007). These proteins are actively degraded in the dark by the E3 ubiquitin ligase COP1 (constitutively photomorphogenic 1) and/or the Cullin4–DDB1 (damaged DNA-binding protein 1)–COP1–SPA (suppressor of *phytochrome A-105*) E3 ubiquitin ligase complex (Osterlund et al. 2000; Seo et al. 2003; Duek et al. 2004; Jang et al. 2005; Yang et al. 2005; Datta et al. 2006, 2007; Laubinger et al. 2006; Liu et al. 2008; H Chen et al. 2010; Chang et al. 2011). Here we show that HMR is another

key positive phy signaling component that accumulates in the light. Unlike the previously reported positive signaling components, though, HMR directly interacts with phys. In addition, all previously characterized signaling components accumulating in the light are themselves transcriptional regulators and likely assert their function by regulating light-dependent gene expression. However, HMR is not a transcription factor, and so far there is no evidence to suggest that HMR regulates these positively acting transcription factors. Rather, HMR appears to regulate the proteolysis of the negatively acting PIFs. Therefore, the signaling pathway from HMR to PIFs provides a mechanism that bridges two light signaling branches: one promoting light responses and one inhibiting them.

The discovery of HMR as a phy signaling component in the nucleus suggests that additional early-acting phy signaling components as well as their signaling mechanisms have been missed from previous molecular genetic studies. Therefore, the elucidation of phy-mediated HMR accumulation as well as its significance in photobody formation and PIF degradation marks only the beginning of our understanding of the early phy signaling mechanisms. Many questions remain to be addressed. The precise biochemical function of HMR in PIF degradation remains unclear. Although it has been proposed that HMR might work like Rad23 in the delivery of multi-ubiquitylated proteins to the proteasome (M Chen et al. 2010), this hypothesis still needs to be further tested. In addition, it is also important to understand how the degradation of HMR itself is regulated, as the level of HMR is restricted in both dark and light conditions. These questions will be the focus of our future investigations. However, the current study is a solid step toward understanding the early phy signaling mechanisms that turn on photomorphogenesis.

Materials and methods

Plant materials

Wild-type Col-0, *phyA-211* (Col-0), and *phyB-9* (Col-0) mutants were used for the characterization of HMR accumulation. Both the PHYB::GFP (PBG) (*Ler*) and the *YHB* (*Ler*) lines have been previously described (Yamaguchi et al. 1999; Su and Lagarias 2007). The *hmr-1* (PBG) and *hmr-1/YHB* (*Ler*) lines were reported by our group (M Chen et al. 2010). The *hmr* TILLING alleles (*hmr-4* to *hmr-13*), including *hmr-5* and *hmr-12*, were backcrossed to Col-0 at least three times before being used for experiments. Supplemental Table S1 lists the stock center accession numbers of the *hmr* TILLING alleles as well as their genotyping CAPS (cleaved amplified polymorphic sequences) (Konieczny and Ausubel 1993) or dCAPS (derived CAPS) (Neff et al. 2002) markers.

Plant growth conditions and hypocotyl measurements

Arabidopsis seed sterilization and stratification as well as standard seedling growth experiments were performed according to the procedures described previously (M Chen et al. 2010). The true-dark and pseudo-dark experiments were performed by following the conditions described by Quail's laboratory (Leivar

et al. 2009). Briefly, seeds were stratified in the dark for 5 d at 4°C. For the true-dark condition, seed germination was induced by the following treatment: 5-min R light pulse ($30 \mu\text{mol m}^{-2} \text{sec}^{-1}$) + 3 h of dark + 5-min FR light pulse ($30 \mu\text{mol m}^{-2} \text{sec}^{-1}$) to inactivate phyB. Then, the seedlings were kept in the dark for 4 d. For the pseudo-dark condition, the only difference was that seed germination was induced using a 5-min R light pulse ($30 \mu\text{mol m}^{-2} \text{sec}^{-1}$) alone, which allowed activated phyB to persist in the dark. Fluence rates of light were measured using an Apogee PS200 spectroradiometer (Apogee Instruments, Inc.). For hypocotyl length measurements, 4-d-old seedlings were scanned using an Epson Perfection V700 photo scanner, and hypocotyls were measured using NIH ImageJ software (<http://rsb.info.nih.gov/nih-image>).

Plasmid construction and generation of transgenic lines

The constructs used for generating recombinant GST fusion proteins were made in the pET42b vector (Novagen). Inserts derived from the *HMR* coding sequence were amplified by PCR using the primers listed in Supplemental Table S3 and ligated into the PstI and EcoRI sites of pET42b. The R293K mutation in *HMR* was generated by site-directed mutagenesis using the primers 5'-CTTTTCAAGGAAGAGAAGCTGCAAGCTGAGCGAG-3' (forward) and 5'-CTCGCTCAGCTTGCAGCTTCTCTTCTTGAAG-3' (reverse). Plasmids containing the *PHYA* and *PHYB* coding sequences used for in vitro transcription and translation were cloned into the pCMX-PL2 vector. Inserts derived from the *PHYA* coding sequence were amplified by PCR and cloned into the XhoI and NheI sites. Likewise, the full-length *PHYB* coding sequence was amplified by PCR and cloned into the KpnI and NheI sites. Primers used for cloning of both *PHYA* and *PHYB* into the pCMX-PL2 vector are listed in Supplemental Table S3.

The *HMR::HA* construct was made by first subcloning full-length *HMR* cDNA into the BglII and SalI sites of the vector pCHF3 (Fankhauser et al. 1999) to generate pCHF3-*HMR*. Then, a DNA fragment encoding $(PT)_4P\text{-}3XHA$ was subcloned into the SalI and PstI sites of pCHF3-*HMR* to generate the pCHF3-*HMR::HA* plasmid. The *HMR::HA* transgenic line was generated by transforming *hmr-5* heterozygous plants with *Agrobacterium tumefaciens* strain GV3101 containing the pCHF3-*HMR::HA* plasmid. The *HMR::HA* transgenic lines were selected on kanamycin plates and screened for homozygous *hmr-5* plants by genotyping.

Protein extraction and Western blot analysis

For *HMR* protein analysis, *Arabidopsis* seedlings grown in the indicated conditions were ground in liquid N₂ and mixed in a 1:2 (milligram to microliter) ratio with extraction buffer containing 100 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1% sodium dodecyl sulfate (SDS), 5 mM ethylenediamine tetraacetic acid (EDTA), 5 mM dithiothreitol (DTT), 10 mM β -mercaptoethanol, 40 μM MG132 (Sigma), 40 μM MG115 (Sigma), and 1 $\times$ EDTA-free protease inhibitor cocktail (Roche). Standard Laemmli sample buffer was added to the crude extract, which was then boiled for 10 min. Cell debris was pelleted by centrifugation at 15,000g for 15 min. The supernatant was considered the total protein extract used for Western analysis.

For PIF3 protein analysis, total protein was extracted according to the protocol described by Huq's laboratory (Shen et al. 2008) with minor changes to the extraction buffer, which contained 100 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5% SDS, 5 mM EDTA, 20% glycerol, 20 mM DTT, 40 mM β -mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM N-ethylmaleimide (NEM), 80 μM MG132, 80 μM MG115, 1 $\times$

EDTA-free protease inhibitor cocktail, and 1 $\times$ phosphatase inhibitor cocktail 3 (Sigma).

Thirty micrograms to 50 μg of protein was resolved by SDS-PAGE, blotted onto a nitrocellulose membrane, probed with the indicated primary antibodies, and then incubated with secondary goat anti-rabbit (Bio-Rad) or goat anti-mouse (Bio-Rad) antibodies conjugated with horseradish peroxidase. The signals were detected by a chemiluminescence reaction using the SuperSignal kit (Pierce). Polyclonal anti-*HMR* antibodies were used at a 1:500 dilution. Monoclonal anti-phyA and anti-phyB antibodies were used at 1:1000 dilutions. Polyclonal anti-RPN6 antibodies (Enzo Life Sciences) were used at a 1:1000 dilution. Polyclonal anti-PIF3 antibodies were used at a 1:500 dilution. Monoclonal anti-HA antibodies (Roche) were used at a 1:1000 dilution to detect *HMR::HA* from plant extracts, and polyclonal anti-HA antibodies (Santa Cruz Biotechnology) were used at a 1:1000 dilution to detect the truncated fragments of phyA-N, phyA-C, and phyA-GP (Fig. 3) in the in vitro pull-down assays. Antibodies against chloroplast ferredoxin-sulfite reductase (SiR) (Chi-Ham et al. 2002) and RNA polymerase II (Abcam clone H5) were used as controls to monitor the purity of the nuclear fractions.

Nuclear fractionation

Seedlings grown in the indicated light conditions were flash-frozen in liquid N₂ and ground to a powder. At a ratio of 4 mL of buffer per gram of fresh weight, the ground tissue was combined with the nuclei extraction buffer containing 20 mM PIPES (pH 6.5), 10 mM MgCl₂, 12% hexylene glycol, 0.25% Triton X-100, 4 mM β -mercaptoethanol, 40 μM MG132, 40 μM MG115, and 1 $\times$ EDTA-free protease inhibitor cocktail. The lysate was filtered through two layers of Miracloth, loaded on top of 2 mL of 25% Percoll (Sigma), and centrifuged at 700g for 5 min at 4°C. The nuclear pellet was dissolved in buffer containing 100 mM Tris HCl (pH 7.5), 100 mM NaCl, 1% SDS, 5 mM EDTA, 5 mM DTT, 10 mM β -mercaptoethanol, 40 μM MG132, 40 μM MG115, and 1 $\times$ EDTA-free protease inhibitor cocktail.

GST pull-down assay

GST pull-down assays were performed according to the procedure described previously (Fankhauser et al. 1999). Briefly, *Escherichia coli* BL21(DE3) cells expressing a given GST fusion protein were harvested by centrifugation at 5000g for 10 min at 4°C. Pelleted cells were lysed by French press in E buffer containing 50 mM Tris HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA), 1% dimethyl sulfoxide (DMSO), 2 mM DTT, and protease inhibitor cocktail (Sigma). Cell debris was pelleted by centrifugation at 10,000g for 20 min at 4°C. The supernatant was further cleared by ultracentrifugation at 50,000g for 15 min at 4°C. GST fusion proteins were purified using glutathione Sepharose beads (GE Healthcare) equilibrated in E buffer. The beads with the immobilized GST fusion proteins were washed four times with E buffer supplemented with 0.1% NP-40.

Apoproteins of phyA and phyB were synthesized using the *pCMX-PL2-PHYA* and *pCMX-PL2-PHYB* plasmids described above and the TNT T7-coupled reticulocyte in vitro transcription and translation kit (Promega) according to the manufacturer's protocol. Holoproteins of phyA and phyB were generated by incubating the respective apoproteins with 20 μM phycocyanobilin (PCB) for 1 h in the dark on ice to allow the incorporation of the chromophore. The in vitro translated phyA or phyB proteins were diluted with E buffer + 0.1% NP-40 and added to the affinity-purified GST fusion protein immobilized on the beads. The mixture was incubated for 2 h under the indicated

conditions. Then, the beads were washed four times with E buffer + 0.1% NP-40. Bound proteins were eluted by boiling in 2× standard Laemmli protein sample buffer.

Coimmunoprecipitation

One gram of 4-d-old seedlings grown in the indicated light condition was ground in liquid N₂, and the powder was resuspended in 2 mL of coimmunoprecipitation buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 2 mM DTT, and 0.1% NP-40, supplemented with 40 μM MG132, 40 μM MG115, and 1× EDTA-free protease inhibitor cocktail. The crude extract was cleared by centrifugation at 20,000g for 20 min at 4°C. The supernatant was further cleared by filtration through a 0.45-μm polyethersulfone filter (VWR International). An aliquot of 1.8 mL of the cleared extract was mixed with 100 μL of anti-HA Affinity Matrix (Roche) and incubated with continuous rotation for 4 h at 4°C. The beads were washed four times with 1 mL of the coimmunoprecipitation buffer, and the immunoprecipitated proteins were eluted with standard 2× Laemmli protein sample buffer.

RNA extraction and quantitative RT-PCR

Total RNA from seedlings of the indicated genotypes and growth conditions was isolated using the Spectrum Plant Total RNA Kit (Sigma). cDNA was synthesized from mRNA using the SuperScript II First-Strand cDNA synthesis kit (Invitrogen) according to the manufacturer's protocol. Quantitative RT-PCR was then performed with FastStart Universal SYBR Green master mix (Roche) in a Mastercycler ep Realplex thermal cycler (Eppendorf). Genes and primer sets used for quantitative RT-PCR are listed in Supplemental Table S4.

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Photoactivated phytochromes interact with HEMERA and promote its accumulation to establish photomorphogenesis in *Arabidopsis*

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