

Light Regulates COP1-Mediated Degradation of HFR1, a Transcription Factor Essential for Light Signaling in Arabidopsis

Jianping Yang,^a Rongcheng Lin,^a James Sullivan,^b Ute Hoecker,^c Bolin Liu,^{a,1} Ling Xu,^a Xing Wang Deng,^b and Haiyang Wang^{a,2}

^a Boyce Thompson Institute for Plant Research, Cornell University, Ithaca, New York 14853

^b Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, Connecticut 06520

^c Department of Plant Developmental and Molecular Biology, Heinrich-heine-Universitaet, D-40225 Duesseldorf, Germany

***Arabidopsis thaliana* seedlings undergo photomorphogenesis in the light and etiolation in the dark. Long Hypocotyl in Far-Red 1 (HFR1), a basic helix-loop-helix transcription factor, is required for both phytochrome A-mediated far-red and cryptochrome 1-mediated blue light signaling. Here, we report that HFR1 is a short-lived protein in darkness and is degraded through a 26S proteasome-dependent pathway. Light, irrespective of its quality, enhances HFR1 protein accumulation via promoting its stabilization. We demonstrate that HFR1 physically interacts with Constitutive Photomorphogenesis 1 (COP1) and that COP1 exhibits ubiquitin ligase activity toward HFR1 in vitro. In addition, we show that COP1 is required for degradation of HFR1 in vivo. Furthermore, plants overexpressing a C-terminal 161-amino acid fragment of HFR1 (CT161) display enhanced photomorphogenesis, suggesting an autonomous function of CT161 in promoting light signaling. This truncated HFR1 gene product is more stable than the full-length HFR1 protein in darkness, indicating that the COP1-interacting N-terminal portion of HFR1 is essential for COP1-mediated destabilization of HFR1. These results suggest that light enhances HFR1 protein accumulation by abrogating COP1-mediated degradation of HFR1, which is necessary and sufficient for promoting light signaling. Additionally, our results substantiate the E3 ligase activity of COP1 and its critical role in desensitizing light signaling.**

INTRODUCTION

As sessile organisms, plants have evolved a high degree of developmental plasticity to optimize their growth and reproduction in response to their environment and various biotic and abiotic stresses. Light is one of the major environmental signals that influences diverse aspects of plant growth and development throughout their life cycle, such as seed germination, seedling deetiolation, gravitropism and phototropism, chloroplast movement, shade avoidance, circadian rhythms, and flowering time (Deng and Quail, 1999; Wang and Deng, 2003).

One of the best-studied plant photoresponses is seedling photomorphogenic development. In the dark, *Arabidopsis thaliana* seedlings undergo skotomorphogenesis (etiolation) and exhibit long hypocotyls, closed cotyledons, and apical hooks and development of the proplastids into etioplasts. By contrast, light-grown seedlings undergo photomorphogenesis (deetiolation) and typically exhibit short hypocotyls, open and expanded

cotyledons, and development of the proplastids into green mature chloroplasts (McNellis and Deng, 1995).

Arabidopsis uses two major classes of photoreceptors to mediate seedling deetiolation. The cryptochromes (cry1 and cry2) absorb the blue/UV-A (320 to 500 nm) light, whereas the phytochromes (phyA to phyE) predominantly regulate responses to red (600 to 700 nm) and far-red light (700 to 750 nm) (Kendrick and Kronenberg, 1994; Briggs and Olney, 2001; Lin, 2002). phyB to phyE predominantly regulate light responses under continuous red and white light, whereas phyA primarily regulates various far-red light responses, including inhibition of hypocotyl elongation, opening of the apical hook, expansion of cotyledons, accumulation of anthocyanin, and far-red light preconditioned blocking of greening (Nagatani et al., 1993; Whitelam et al., 1993; Neff et al., 2000).

Molecular and genetic studies in *Arabidopsis* have identified numerous light signaling intermediates, including both positive and negative regulators of light signaling (for review, see Quail, 2002; Wang and Deng, 2004). Notably, a group of proteins named Constitutive Photomorphogenic/De-Etiolated/Fusca (COP/DET/FUS) function downstream of multiple photoreceptors (including both phytochromes and cryptochromes) to repress photomorphogenesis (Wei and Deng, 1996). Among them, the RING finger protein Constitutive Photomorphogenesis 1 (COP1) seems to be a key regulator (Deng et al., 1992). It was shown that COP1 possesses E3 ligase activity toward a group of photomorphogenesis-promoting factors, including HY5, LAF1, and phyA, and is responsible for their targeted degradation, thus

¹ Current address: Department of Biology, University of Montreal, Montreal, Quebec, Canada H1X 2B2.

² To whom correspondence should be addressed. E-mail hw75@cornell.edu; fax 607-254-1242.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Haiyang Wang (hw75@cornell.edu).

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desensitizing light signaling (Osterlund et al., 2000; Saijo et al., 2003; Seo et al., 2003, 2004).

In addition to these negative regulators of light signaling, one positive regulator, HY5, also acts downstream of both phytochromes and cryptochromes (Oyama et al., 1997). *HY5* encodes a basic domain/leucine zipper transcription factor whose abundance is correlated with the extent of photomorphogenesis (Osterlund et al., 2000). Moreover, many signaling components specific for individual photoreceptors have been identified, and most of them have been characterized at the molecular level. Among them, PIF3, NDPK2, PKS1, COG1, PFT1, and PRR7 are shared by both phyA and phyB; GI, ELF3, ELF4, ARR4, PIF4, and SRR1 are specific for phyB signaling; and FHY1, FHY3, FAR1, PAT1, Long Hypocotyl in Far-Red 1 (HFR1), LAF1, LAF3, LAF6, FIN219, SPA1, and EID1 are specific for phyA signaling. SUB1, a Ca²⁺ binding protein, represses cryptochrome signaling and modulates phytochrome signaling, whereas PP7 is a positive regulator of blue light signaling (for review, see Quail, 2002; Wang and Deng, 2004). At present, the biochemical functions and the mechanisms dictating the specificity of these signaling molecules are largely unknown.

HFR1/REP1/RSF1 (hereafter, we used *HFR1*) was originally identified as a positive regulator of phyA signaling. It encodes an atypical basic helix-loop-helix (bHLH) transcription factor of 292 amino acids. *hfr1* loss-of-function mutants are defective in a subset of phyA-mediated far-red light responses, including inhibition of hypocotyl elongation, suppression of hypocotyl negative gravitropism, and induction of *CAB* (encoding chlorophyll *a/b* binding protein) gene expression, without affecting far-red light preconditioned blocking of greening and induction of *CHS* (encoding chalcone synthase) gene expression (Fairchild et al., 2000; Fankhauser and Chory, 2000; Soh et al., 2000). More recently, it was reported that *hfr1* alleles also have reduced deetiolation responses when grown in blue light, including hypocotyl elongation, cotyledon opening, and anthocyanin accumulation. The analysis of double mutants between *hfr1* and different blue light photoreceptor mutants demonstrated that, in addition to its role in phyA signaling, HFR1 is a component of cry1-mediated blue light signaling (Duek and Fankhauser, 2003). Thus, HFR1 may represent a point of signal integration from phyA and cry1, either as a convergence of two independent signaling pathways or as a result of interaction of phyA and cry1 at the photoreceptor molecule level (Ahmad et al., 1998).

It was previously demonstrated that *HFR1* mRNA levels are high in seedlings grown under continuous darkness, far-red, and blue light, whereas *HFR1* transcript levels are very low in seedlings grown in continuous red light, irrespective of their fluence rates (Fairchild et al., 2000; Soh et al., 2000; Duek and Fankhauser, 2003). Thus, light-quality control of *HFR1* transcript levels could serve as a mechanism for specifying HFR1 activity in far-red and blue light signaling. Kinetic analysis of *HFR1* accumulation demonstrated that *HFR1* transcript levels increase in response to far-red or blue light, but decrease in response to red or white light. However, the levels of *HFR1* transcript change slowly. *HFR1* mRNA levels are not apparently affected by a 2-h red light treatment, slight reductions are seen in seedlings treated with red light for 4 h, and there is a twofold decline after 18 h of red light treatment. Prolonged red light treatment is required to further

reduce *HFR1* transcript levels (Duek and Fankhauser, 2003). Considering the rapidity of plant responses to changes in their light environment, the slow kinetics of *HFR1* transcript level change might not be the sole or primary mechanism for regulating HFR1 activity. Consistent with this notion, a recent report showed that transgenic plants overexpressing a full-length *HFR1* gene display no apparent phenotypic alternations under far-red light (Yang et al., 2003), supporting the notion that HFR1 activity might be regulated at the translational or posttranslational level.

In this study, we show that the level of HFR1 protein correlates with the magnitude of Arabidopsis seedling photomorphogenesis and is a key regulator of this process. We demonstrate that HFR1 is a short-lived protein in darkness and its accumulation is enhanced by light. Regulated degradation of HFR1 is mediated by the 26S proteasome. We present genetic, molecular, and biochemical evidence to support that COP1 is an E3 ubiquitin ligase targeting HFR1 for degradation, thus desensitizing light signaling. Together with previously reported data, our study suggests that control of HFR1 activity in light signaling involves regulation of its message RNA and protein at the posttranscriptional and posttranslational levels.

RESULTS

Overexpression of Arabidopsis HFR1 Causes Enhanced Photomorphogenesis

To further investigate the function and regulation of Arabidopsis *HFR1* in light signaling, we generated transgenic plants overexpressing a nine-copy of c-myc epitope tagged full-length *HFR1* (*Myc-HFR1*; driven by the constitutive, strong 35S promoter of *Cauliflower mosaic virus*) in both wild-type and *hfr1-201* mutant (a putative null allele; Soh et al., 2000) backgrounds. More than forty independent transgenic lines were obtained in each of the backgrounds. When grown in various light conditions (darkness, far red, red, and blue), all transgenic lines in the wild-type background possessed hypocotyls of similar lengths to those of wild-type control plants (data not shown). This result is consistent with a recent report that transgenic plants overexpressing an *HFR1* full-length gene (without any epitope tags) have no obvious phenotypic effects under far-red light, even though high levels of *HFR1* mRNA accumulation were observed (Yang et al., 2003). However, the *Myc-HFR1* transgene can largely rescue the long hypocotyl phenotype of *hfr1-201* mutants under far-red and blue light conditions, suggesting that the *Myc-HFR1* transgene is biologically functional. Interestingly, these transgenic plants exhibited a hypersensitive response to red light inhibition of hypocotyl growth (Figures 1A and 1B).

In a parallel effort, we generated transgenic plants overexpressing a green fluorescent protein (GFP) tagged full-length *HFR1* (*GFP-HFR1*) gene. Strikingly, these transgenic plants exhibited much more enhanced photomorphogenesis (with drastically shorted hypocotyls) in response to far-red, red, and blue light, even though they etiolated normally in darkness (Figures 1A and 1B). In addition, the *GFP-HFR1* transgenic plants, but not the *Myc-HFR1* transgenic plants, displayed increased expression of both *CAB3* and *RBCS* (encoding the

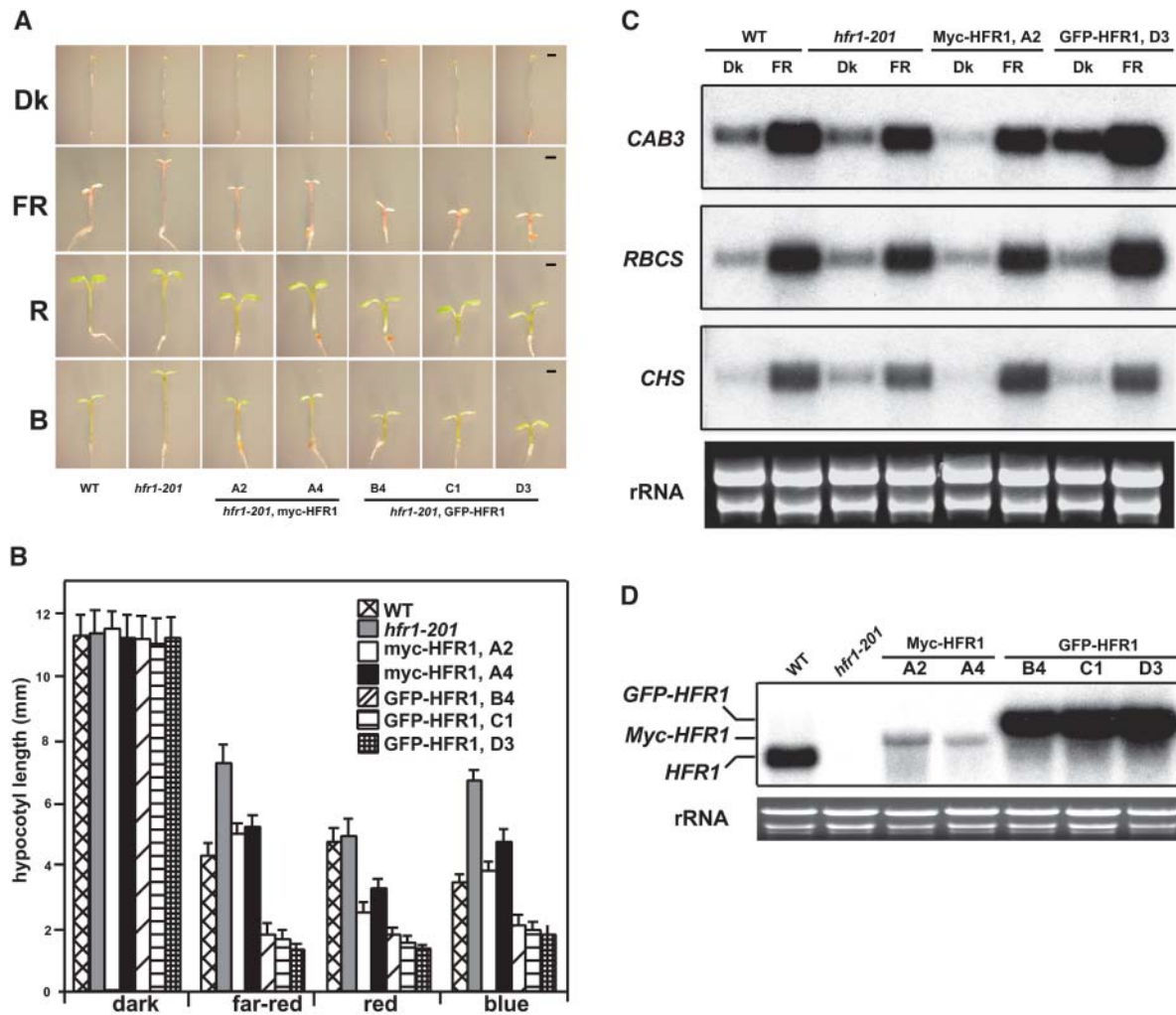


Figure 1. Hypersensitive Light Responses of HFR1 Overexpression Lines.

(A) Morphology of Arabidopsis seedlings overexpressing *Myc-HFR1* or *GFP-HFR1* grown under various light conditions. Dk, darkness; FR, far-red; R, red; B, blue light. Photographs of seedlings from each light condition were taken at the same magnification. Bars = 1 mm.

(B) Quantification of hypocotyl lengths (average of 20 seedlings) under various light conditions. Bars stand for standard deviations.

(C) Light-responsive gene expression (*CAB3*, *RBCS*, and *CHS*). Seedlings were grown in darkness (Dk) for 4 d then transferred into far-red light (FR) for 12 h. An rRNA UV fluorescence image of the duplicating gels was shown as a loading control. Transgenic line A2 and transgenic line D3 were used as the representative lines for *Myc-HFR1* and *GFP-HFR1*, respectively, unless otherwise indicated.

(D) RNA gel blot analysis of *Myc-HFR1* and *GFP-HFR1* transgene expression. Seedlings were grown under continuous far-red light for 5 d. An rRNA UV fluorescence image of the gel was shown as a loading control.

small subunit of ribulose-1,5-bisphosphate carboxylase) genes in response to far-red light induction. *CHS* expression was not obviously affected in either of the transgenic plants (Figure 1C), suggesting that HFR1 plays an important role in regulating *CAB* and *RBCS*, but not *CHS* expression. RNA gel blot analysis with seedlings grown under continuous far-red light showed that the *GFP-HFR1* transgenic lines accumulated high levels of transcript as expected (approximately twofold to threefold higher than the endogenous *HFR1* transcript); however, the *Myc-HFR1* transgenic lines accumulated low levels of transcript for unknown reasons (Figure 1D). Thus, the differential light responses displayed by the *Myc-HFR1* and *GFP-HFR1* transgenic plants could

be due to different expression levels of the transgenes and/or differential effects of the Myc and GFP tags on the stability or activity of HFR1 protein. Nonetheless, the increased light responsiveness observed with the *GFP-HFR1* transgenic lines indicates that HFR1 is necessary and sufficient for light signaling.

Light Enhances HFR1 Protein Accumulation

To determine whether the level of HFR1 protein correlates with the extent of seedling photomorphogenesis, *Myc-HFR1* and *GFP-HFR1* transgenic seedlings were grown under various fluence rates (light intensities) of white light for 4 d. As shown in

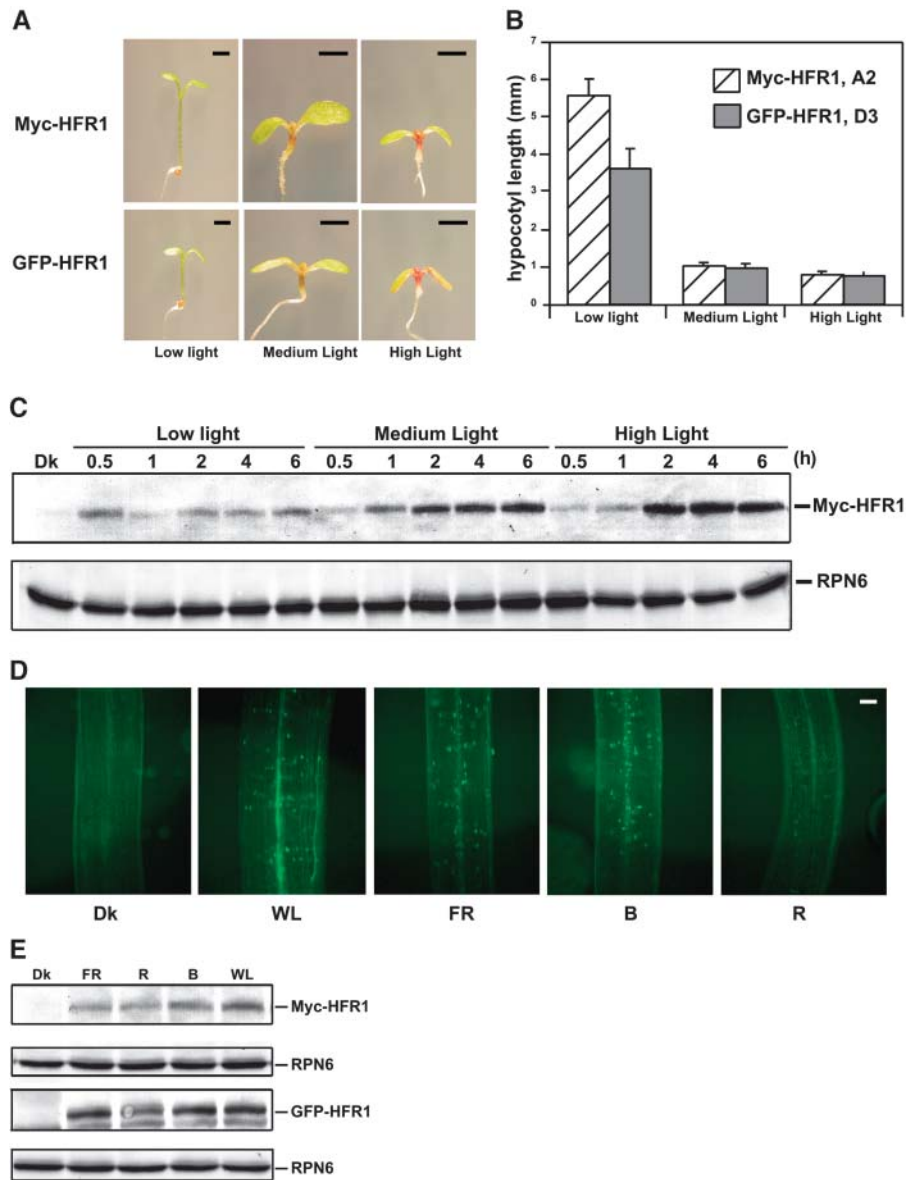


Figure 2. Light Enhances HFR1 Protein Accumulation.

(A) Morphology of 4-d-old *Myc-HFR1* (top) and *GFP-HFR1* (bottom) transgenic seedlings grown under various fluence rates of white light. Seedlings shown in the left panels were grown under low light intensity ($\sim 10 \mu\text{mol}/\text{m}^2\text{s}$), seedlings shown in the middle panels were grown under medium light intensity ($\sim 100 \mu\text{mol}/\text{m}^2\text{s}$), and the seedlings shown in the right panels were grown under high light intensity ($\sim 500 \mu\text{mol}/\text{m}^2\text{s}$). Bars = 1 mm.

(B) Quantification of hypocotyl lengths of the seedlings grown under various fluence rates of white light. Bars stand for standard deviations.

(C) Immunoblot analyses of Myc-HFR1 in seedlings grown in continuous darkness (Dk) for 4 d and then transferred to white light of different fluence rates for the indicated amounts of time. An anti-RPN6 (a 26S proteasome subunit) immunoblot is shown below to indicate approximately equal loadings.

(D) Enhanced GFP-HFR1 fluorescence and nuclear accumulation (confirmed by 4',6-diamidino-2-phenylindole staining; data not shown) in hypocotyls cells under white light (WL) for 4 h or far-red (FR), red (R), and blue (B) light for 6 h. Dk, dark-grown seedlings. Photographs of seedlings were taken at the same magnification. Bar = 50 μm .

(E) Various colors of light enhance HFR1 accumulation during dark-to-light transition. Arabidopsis seedlings expressing *Myc-HFR1* or *GFP-HFR1* were grown in darkness (Dk) for 4 d and then transferred to various light conditions for 6 h before total protein was extracted for immunoblot analysis. Immunoblots of anti-RPN6 were shown below to indicate approximately equal loadings. FR, far-red; R, red; B, blue; WL, white light.

Figures 2A and 2B, seedlings grown in higher light intensities displayed stronger photomorphogenic phenotypes (such as shorter hypocotyls and increased accumulation of anthocyanin). An immunoblot analysis was conducted to determine the effects of fluence rates on HFR1 protein accumulation. Surprisingly, both fusion proteins were barely detectable under these light conditions (data not shown). Additional immunoblot analyses also failed to detect Myc-HFR1 and GFP-HFR1 fusion proteins in 4-d-old seedlings grown under different continuous light conditions (darkness, far-red, red, or blue light) (data not shown), suggesting that these HFR1 fusion proteins might be unstable. Next, we performed immunoblot analyses with *Myc-HFR1* transgenic seedlings grown in darkness for 4 d and then transferred to white light of different light intensities for different amounts of time (0.5, 1, 2, 4, and 6 h). As shown in Figure 2C, Myc-HFR1 was rapidly induced by light treatment and became clearly detectable after half an hour of light treatment. Faster and higher Myc-HFR1 accumulation was observed in seedlings grown under high fluence rates. It took ~ 2 h of light induction for Myc-HFR1 accumulation to reach the peak, and its levels did not change significantly until 6 h after the induction (Figure 2C). However, Myc-HFR1 protein levels started to decline 12 h after the induction, becoming barely detectable after 24 h (data not shown). Thus, the more rapid and higher accumulation of Myc-HFR1 induced by higher light intensities correlates with the stronger photomorphogenic phenotypes of Arabidopsis seedlings, pointing to HFR1 as a key determinant of the degree of Arabidopsis seedling photomorphogenesis. In addition, the observation that Myc-HFR1 was rapidly induced by light treatment but that its levels decline after prolonged light exposure suggests that HFR1 might be only required for the initial transition from dark-adapted development to light-adapted development.

To confirm the observation that light enhances HFR1 protein accumulation during dark-to-light transition, we monitored GFP-HFR1 fluorescence levels in the *GFP-HFR1* transgenic seedlings using a fluorescence microscope. GFP-HFR1 fluorescence was barely detectable in dark-grown seedlings. Clear nuclear accumulation of GFP-HFR1 was detected in seedlings treated with white light for 2 h or treated with monochromatic far-red, red, or blue light for 4 h. No obvious cytoplasmic fluorescence for GFP-HFR1 was observed under all tested light conditions (data not shown). Kinetic studies indicated that it required ~ 4 h of white light treatment for the GFP-HFR1 nuclear fluorescence to reach its peak level. Longer treatments (6 to 8 h) were needed to reach similar levels with monochromatic far-red, red, or blue light (Figure 2D); however, the GFP-HFR1 nuclear fluorescence was apparently weaker in red light-treated samples compared with samples treated with other colors of light (white, far-red, or blue light) (Figure 2D). Prolonged light treatment apparently did not promote further nuclear accumulation of GFP-HFR1 (data not shown). It should be noted that for this assay, the applied fluence rate of red light was almost 60-fold and 6-fold higher than those of far-red and blue light, respectively (see Methods). Thus, the apparently weaker GFP-HFR1 fluorescence observed in seedlings exposed to red light suggests that red light is not as effective as far-red and blue light in promoting GFP-HFR1 accumulation. An immunoblot analysis also supported the conclusion that

light, irrespective of the wavelength, enhances Myc-HFR1 and GFP-HFR1 protein accumulation in seedlings transferred from darkness to different light conditions (far-red, red, blue, and white light) (Figure 2E).

HFR1 Is Rapidly Degraded through a 26S Proteasome-Dependent Pathway

To test whether the low levels of HFR1 in dark-grown seedlings might be due to HFR1 degradation mediated by the 26S proteasome, we tested the effects of a proteasome inhibitor, MG132, on HFR1 protein accumulation. Arabidopsis seedlings expressing *Myc-HFR1* or *GFP-HFR1* were grown in darkness for 4 d and then treated with MG132 or mock treated with 0.1% DMSO for 6 h. Total protein was extracted and subjected to immunoblot analysis. As shown in Figures 3A and 3B, MG132 treatment drastically increased Myc-HFR1 and GFP-HFR1 accumulation in dark-grown seedlings, indicating that HFR1 protein is subject to 26S proteasome-mediated proteolysis in the dark. Fluorescence microscope observation also confirmed that MG132, but not DMSO, promoted GFP-HFR1 nuclear accumulation (Figure 3C).

We next performed a time-course study to compare the degradation kinetics of HFR1 protein under darkness and in white light conditions. Arabidopsis seedlings expressing *Myc-HFR1* or *GFP-HFR1* were grown in darkness for 4 d and then treated with MG132 for 24 h to promote accumulation of the fusion proteins. Next, the seedlings were washed three times with liquid MS medium to remove MG132 and then incubated under darkness or continuous white light for 1, 2, 4, 6, 8, 12, and 24 h. Total protein was extracted and subjected to immunoblot analysis. As shown in Figure 3D, 1 h after the removal of MG132, much higher levels of Myc-HFR1 or slightly higher levels of GFP-HFR1 were detected compared with the start points, possibly because of the lag effect of MG132. Under dark conditions, levels of Myc-HFR1 dropped rapidly during the interval between hour 1 and hour 2 (approximately reduced by half) and became undetectable 24 h after the removal of MG132. Under white light conditions, degradation of Myc-HFR1 seems to be slower, and small amounts of Myc-HFR1 were still detected 24 h after the removal of MG132. Degradation of GFP-HFR1 appears to be slower than that of Myc-HFR1 because GFP-HFR1 was still detected 24 h after the removal of MG132 under both darkness and white light conditions. This result suggests that GFP-HFR1 might be more stable than Myc-HFR1, particularly under dark conditions.

We next compared the degradation kinetics of HFR1 under different colors of light to test the effect of light quality on promoting HFR1 accumulation. *Myc-HFR1* transgenic plants were first grown in continuous red light for 4 d and then treated with MG132 for 24 h to promote Myc-HFR1 protein accumulation. Next, the seedlings were transferred to darkness, continuous far-red, red, or blue light for 6, 24, and 48 h, in the presence of DMSO or MG132. As shown in Figure 4A, in samples mock treated with DMSO, Myc-HFR1 was completely degraded by 6 h in darkness, but was clearly visible in light-grown seedlings. Twenty-four hours after the transfer, Myc-HFR1 was barely detectable in seedlings grown in red light, but was still abundant in seedlings grown in far-red or blue light conditions. Forty-eight hours after the transfer, trace amounts of Myc-HFR1 were still

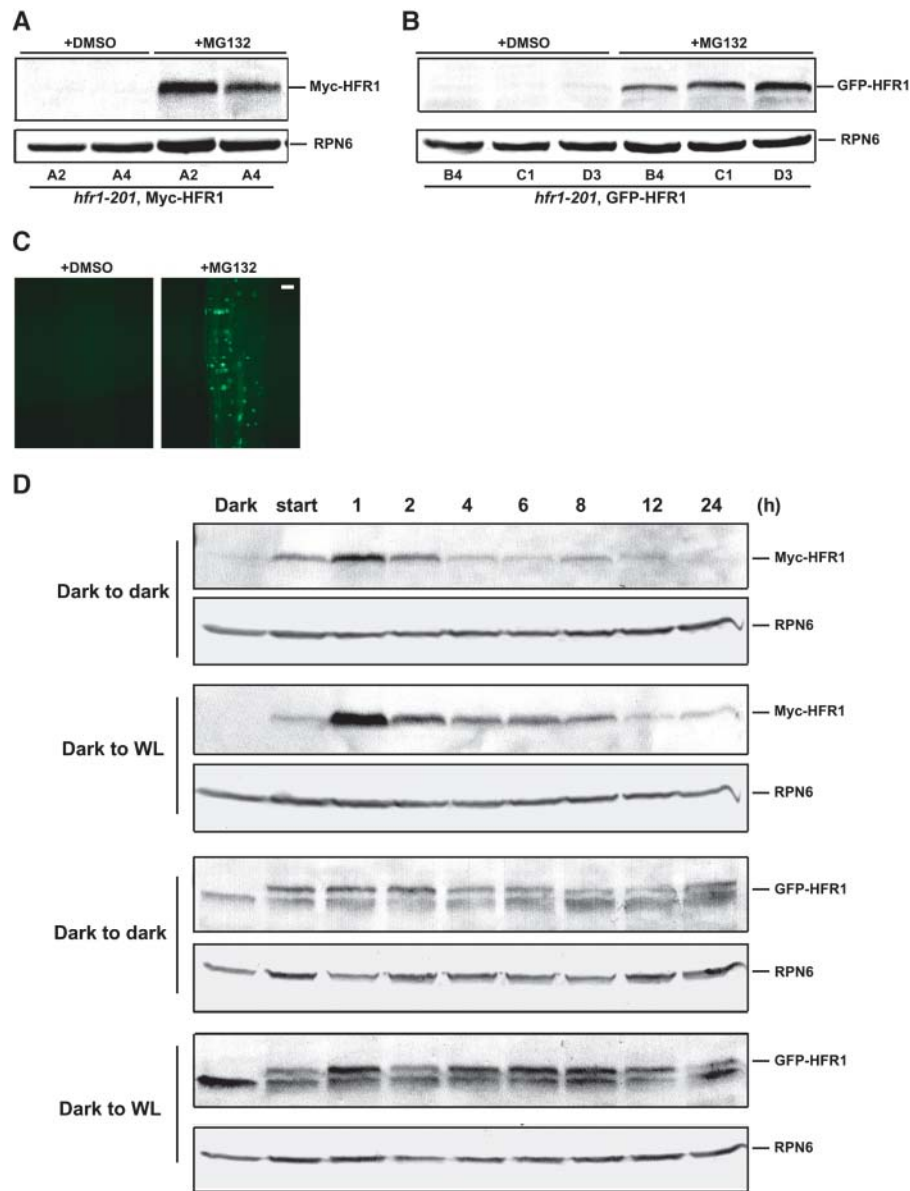


Figure 3. HFR1 Protein Is Rapidly Degraded through a Proteasome-Dependent Pathway.

(A) and **(B)** Myc-HFR1 and GFP-HFR1 proteins are stabilized by the proteasome inhibitor MG132. Arabidopsis seedlings expressing *Myc-HFR1* or *GFP-HFR1* were grown in darkness for 4 d and then treated with MG132 or mock treated with 0.1% DMSO for 6 h. Total protein was extracted and subjected to immunoblot analysis.

(C) Fluorescence images of hypocotyl cells of Arabidopsis seedlings expressing *GFP-HFR1*. Seedlings were grown in darkness for 4 d and then treated with MG132 or mock treated with 0.1% DMSO for 24 h. Photographs of seedlings were taken at the same magnification. Bar = 50 μ m.

(D) Arabidopsis seedlings expressing *Myc-HFR1* or *GFP-HFR1* were grown in darkness for 4 d and then treated with MG132 for 24 h to promote accumulation of the fusion proteins. The seedlings were then washed thoroughly and incubated in darkness or white light (WL) for indicated amounts of time. Total protein was extracted and subjected to immunoblot analysis. Immunoblots of anti-RPN6 were shown below to indicate approximately equal loadings.

seen in seedlings grown in blue light but not in seedlings grown in other light conditions. MG132 treatment stabilized Myc-HFR1 under all light conditions. In the presence of MG132, Myc-HFR1 was clearly detectable in seedlings grown in darkness for 6 h. Twenty-four hours after the transfer, small amounts of Myc-

HFR1 were detected in dark-grown seedlings, and significantly higher levels of Myc-HFR1 were detected in seedlings grown in far-red, red, or blue light conditions. Forty-eight hours after the transfer, Myc-HFR1 became undetectable in seedlings grown in red light, barely detectable in far-red light-grown seedlings, but

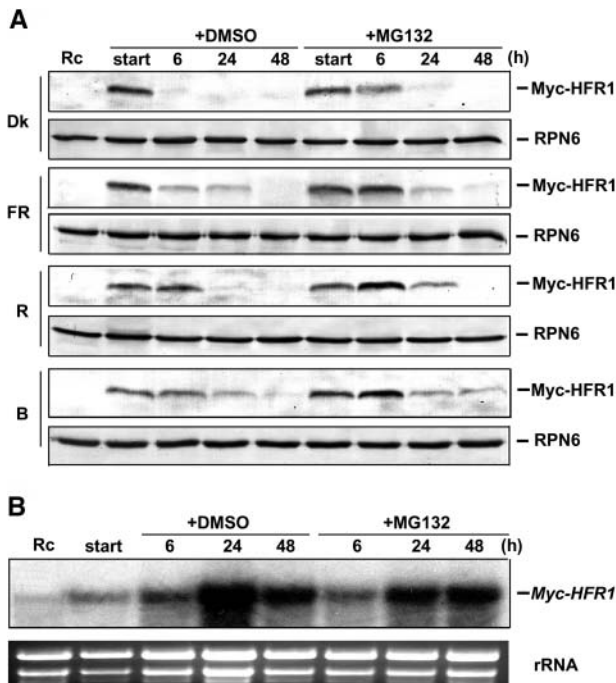


Figure 4. Light-Quality Control of HFR1 Protein Accumulation.

(A) Differential effects of light qualities on HFR1 protein accumulation. Arabidopsis seedlings expressing Myc-HFR1 were grown in continuous red light (Rc) for 4 d and then treated with MG132 for 24 h to promote the accumulation of HFR1 protein (start point). The seedlings were then incubated in darkness in the presence of DMSO or MG132 for the indicated amounts of time before harvested for immunoblot analyses. Immunoblots of anti-RPN6 were shown below to indicate approximately equal loadings.

(B) Effects of DMSO and MG132 on *Myc-HFR1* transcript accumulation. Arabidopsis seedlings were grown in continuous red light (Rc) for 4 d and then treated with MG132 for 24 h. The seedlings were then transferred to darkness for the indicated times in the presence of DMSO or MG132 before total RNAs were extracted for RNA gel blot analysis. A UV fluorescence image of rRNA of the gel is shown as a loading control.

was still clearly detected in blue light-grown seedlings (Figure 4A). This result further substantiates that HFR1 is a shorted-lived protein in darkness (took <6 h to degrade) and that light, irrespective of the wavelength, promotes the stabilization of HFR1. It should be noted that seedlings used in this assay were grown under continuous red light for 4 d before being subjected to MG132 treatment. The faster degradation kinetics of Myc-HFR1 protein from seedlings grown in continuous red light (this assay) than Myc-HFR1 protein derived from dark-grown seedlings (see Figure 3D) suggests that Myc-HFR1 proteins from seedlings grown under different light conditions differ in their stability. More detailed experiments are required to unequivocally determine the differential effects of light qualities and fluence rates on promoting HFR1 protein accumulation.

To exclude the possibility that the increased HFR1 protein accumulation resulting from MG132 treatment might be due to increased transcript accumulation conferred by MG132, we conducted an RNA gel blot analysis to determine the effects of

MG132 on *Myc-HFR1* transcript levels. *Myc-HFR1* transgenic plants were grown in continuous red light for 4 d and then treated with MG132 for 24 h. Next, the seedlings were transferred to darkness (in the presence of DMSO or MG132) for different times. As shown in Figure 4B, *Myc-HFR1* transcript levels were very low from continuous red light-grown seedlings, and MG132 treatment slightly increased its transcript levels. During the incubation in darkness, *Myc-HFR1* transcript levels continued to increase and remained high in the presence of both MG132 and DMSO. Slightly higher *Myc-HFR1* transcripts levels were observed at 6 and 24 h after the transfer to darkness in the presence of DMSO. The contrasting effects of DMSO and MG132 on *Myc-HFR1* transcript and protein accumulation supports the claim that the increased accumulation of HFR1 protein by MG132 is most likely a result of stabilizing HFR1 protein by blocking 26S proteasome activity, rather than by increasing *HFR1* transcript accumulation.

HFR1 Physically Interacts with COP1

To look for the E3 ubiquitin ligase(s) responsible for targeting HFR1 for degradation in darkness, we tested whether HFR1 is capable of interacting with COP1, a photomorphogenesis repressor recently shown to possess E3 activity toward HY5, LAF1, and phyA (Saijo et al., 2003; Seo et al., 2003, 2004). First, we tested their interactions using a yeast two-hybrid assay. We found that full-length COP1 was capable of interacting with full-length HFR1, an N-terminal 189-amino acid fragment of HFR1 (NT189), and a deletion derivative of HFR1 lacking the bHLH domain (Figure 5A). These results suggest that the N-terminal portion upstream of the bHLH domain of HFR1 (N-terminal 131-amino acid fragment of HFR1, NT131) is responsible for interacting with COP1. Full-length HFR1 also interacted with full-length COP1 and several mutant derivatives of COP1, including deletion mutants lacking either the RING finger or the coil domain or a deletion mutant lacking both the RING finger and coil domains. Strong interaction was also observed between HFR1 with the coiled-coil domain of COP1. No interaction was detected between HFR1 and the N-terminal 282-amino acid fragment of COP1 (N282) that contains both the RING finger and coiled-coil domains or the WD-repeat domain of COP1. Thus, the interaction between COP1 and HFR1 appears to involve both the coiled-coil region and the WD-repeat domain of COP1 (Figure 5B). Interestingly, a small deletion or a single amino acid substitution within the WD-repeat domain of COP1 (corresponding to the molecular lesions in *cop1-8* and *cop1-9* mutants, respectively), which causes a lethal *cop1* mutant phenotype (McNellis et al., 1994), abolished the COP1-HFR1 interaction, supporting a physiological relevance of COP1-HFR1 physical interaction.

To verify these protein-protein interactions, we conducted an in vitro interaction assay using recombinant proteins (Hoecker and Quail, 2001). COP1 and GAL4 activation domain (GAD)-tagged HFR1 were synthesized by coupled in vitro transcription and translation. A coimmunoprecipitation was performed with antibodies against GAD. Approximately 6% of the COP1 protein or ~2% of mutant COP1 lacking the coil domain added to the assay were coprecipitated by GAD-HFR1, whereas <0.1% of the

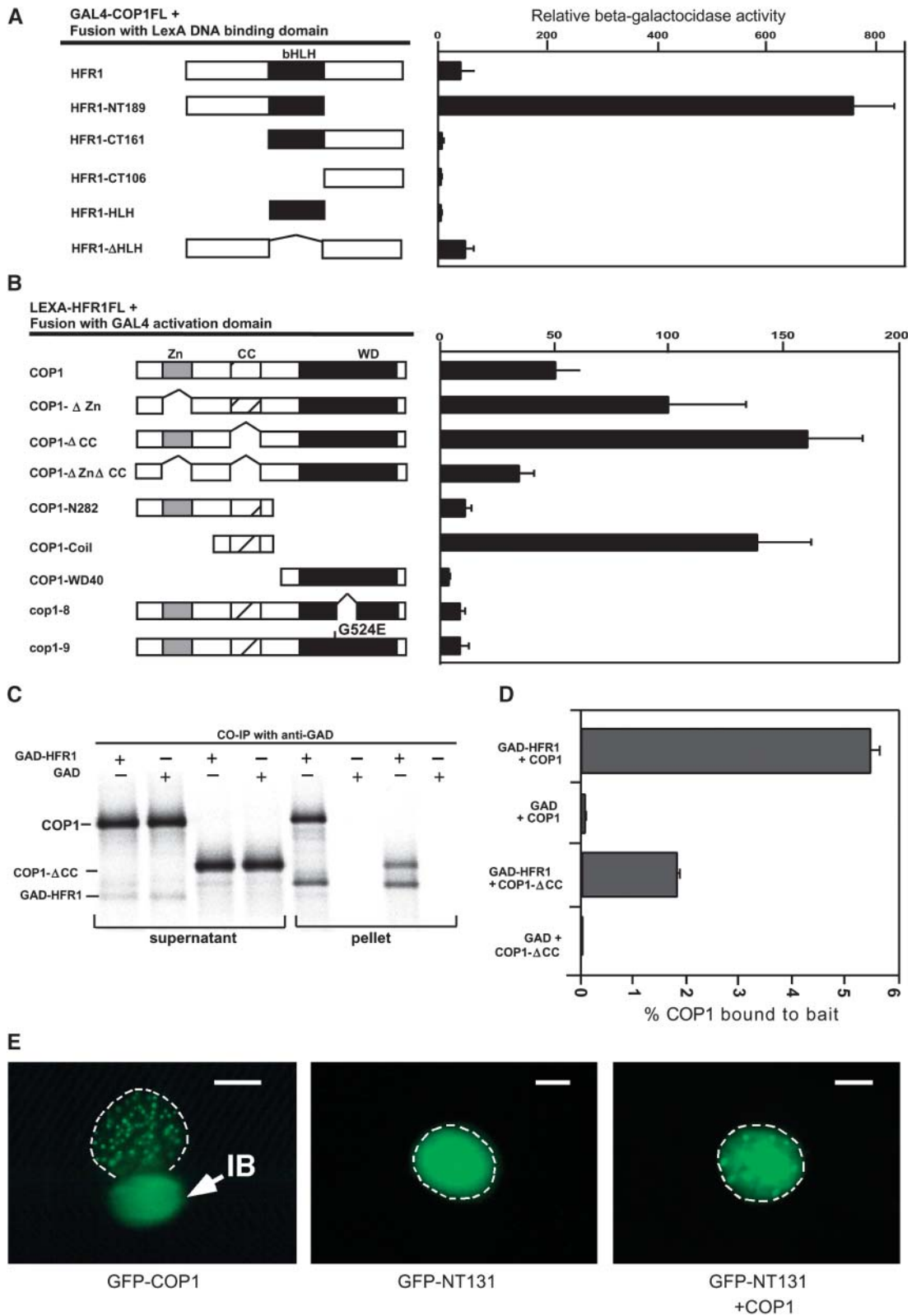


Figure 5. HFR1 Physically Interacts with COP1.

(A) and (B) HFR1-COP1 interaction analyzed by the yeast two-hybrid assay. Left panels illustrate the prey and bait constructs, and the right panels

added COP1 or its coil deletion mutant were precipitated by the control bait GAD (Figures 5C and 5D).

To further test COP-HFR1 interaction *in vivo*, we conducted a living onion cell colocalization assay of HFR1 and COP1. We fused the putative COP1-interacting domain of HFR1, the N-terminal 131-amino acid fragment of HFR1 (NT131), with GFP and introduced the GFP-NT131 transgene into onion epidermal cells via particle bombardment. As shown in Figure 5E, GFP-NT131 was exclusively and uniformly distributed in the nucleus. Sequence analysis of HFR1 revealed that NT131 contains a stretch of basic amino acids (KKRR) that could serve as a potential nuclear targeting signal. As previously reported, GFP-COP1 exhibits punctuate green fluorescent speckles over the weak and uniform green fluorescent background in the nuclei as well as cytoplasmic inclusion bodies (Ang et al., 1998; Wang et al., 2001). Strikingly, coexpression of GFP-NT131 with an untagged COP1 resulted in bright green nuclear speckles over the uniform green fluorescence background, a characteristic pattern of GFP-COP1 fusion protein (Figure 5E). This result demonstrates that GFP-NT131 can be recruited into the nuclear speckles of COP1, supporting a direct interaction between COP1 and HFR1 in living plant cells.

COP1 Ubiquitinates HFR1 *in Vitro*

Recently, it was shown that COP1 functions as an E3 ubiquitin ligase that ubiquitinates itself, HY5, LAF1, and phyA using *in vitro* ubiquitination assays (Saijo et al., 2003; Seo et al., 2003, 2004). The observed physical interaction between HFR1 and COP1 prompted us to test whether HFR1 might be another substrate subject to COP1-mediated ubiquitination. We conducted an *in vitro* ubiquitination assay in which we combined recombinant rabbit E1 (Boston Biochem), rice (*Oryza sativa*) Rad6 (Yanagawa et al., 2004) as the E2, maltose binding protein (MBP) fusion of COP1 as the E3, and a recombinant fusion protein of glutathione S-transferase (GST)-tagged HFR1 N-terminal 131-amino acid fragment (GST-NT131) as the substrate, together with biotinylated-ubiquitin (Affiniti), to assay the formation of ubiquitinated HFR1 in a buffer containing Zn^{2+} ions (which seems to be required for COP1 function, consistent with its structural feature, a RING motif protein). GST-NT131 was used in the assay for two reasons. First, NT131 of HFR1 is involved in interacting with COP1 (Figure 5E); second, recombinant GST-NT131 protein produced in *Escherichia coli* is much more soluble and easier to

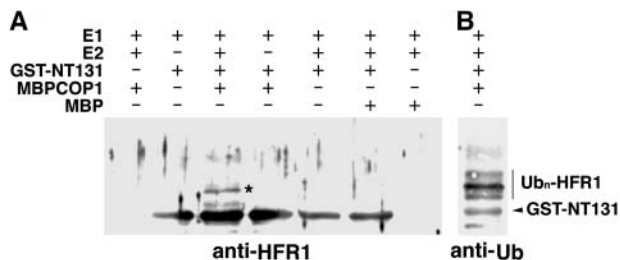


Figure 6. *In Vitro* Ubiquitination Assay of HFR1.

GST-tagged NT131 of HFR1 (GST-NT131) protein was used as the substrate.

(A) An anti-HFR1 immunoblot showing ubiquitinated GST-NT131 (indicated by an asterisk).

(B) An immunoblot showing ubiquitinated HFR1 detected by streptavidin-conjugated horseradish peroxidase for biotinylated ubiquitin, followed by chemiluminescence visualization. The arrowhead indicates unmodified GST-NT131.

purify than the GST-tagged full-length HFR1 protein (data not shown). As shown in Figure 6A, the addition of E1, E2, and MBP-COP1, together with GST-NT131, resulted in the formation of a higher molecular weight band (indicated by an asterisk), which likely represents ubiquitinated GST-NT131. A separate immunoblot analysis with antiubiquitin antibodies confirmed the formation of oligo-ubiquitinated GST-NT131 (Figure 6B), supporting the potential ability of COP1 to add ubiquitins to HFR1.

HFR1 Degradation *In Vivo* Is COP1 Dependent

To substantiate that the E3 activity of COP1 toward HFR1 is physiologically relevant *in vivo*, we introduced the *GFP-HFR1* transgene into two weak *cop1* mutant alleles, *cop1-4* and *cop1-6*, to analyze their genetic interactions. *cop1-4* encodes a truncated *COP1* gene product terminating at amino acid 282, whereas the *cop1-6* allele has an in-frame five-amino acid insertion between codons 301 and 302 (McNellis et al., 1994). Both *cop1-4* and *cop1-6* are considered weak alleles of *cop1* (null alleles of *cop1* are seedling lethal). As shown in Figures 7A and 7B, *cop1* mutants harboring the *GFP-HFR1* transgene exhibited enhanced photomorphogenesis under all light conditions (including darkness, far red, red, and blue), and the enhancement was particularly obvious for seedlings grown in the

Figure 5. (continued).

show the corresponding β -galactosidase activities. The value is the average of six individual yeast colonies, and the error bars represent the standard deviations.

(C) *In vitro* coimmunoprecipitation of COP1 by HFR1. The 35S-labeled COP1 or COP1- Δ CC were incubated with partially labeled GAD-HFR1 or GAD and coimmunoprecipitated with anti-GAD antibodies. Supernatant fractions (1.6%) and pellet fractions (33.3%) were resolved by SDS-PAGE and visualized by autoradiography using a phosphor imager. Successful immunoprecipitation of GAD was confirmed on a separate gel (data not shown).

(D) Quantification of the fractions of bound prey proteins. Error bars denote one standard error of the mean of two replicate experiments.

(E) Recruitment of GFP-NT131 into COP1 nuclear speckles in living onion epidermal cells. The left panel shows that GFP-COP1 is localized in bright nuclear speckles and the fluorescent cytoplasmic inclusion body (IB; indicated by an arrow). The middle panel shows that GFP-NT131 is uniformly distributed in the nucleus. Coexpression of GFP-NT131 with nontagged COP1 resulted in bright green nuclear speckles over the uniform green fluorescent background. This result suggests a direct interaction between COP1 and HFR1 in living plant cells. Dashed lines demarcate the nuclei (confirmed by 4',6'-diamidino-2-phenylindole staining; data not shown). Bars = 50 μ m.

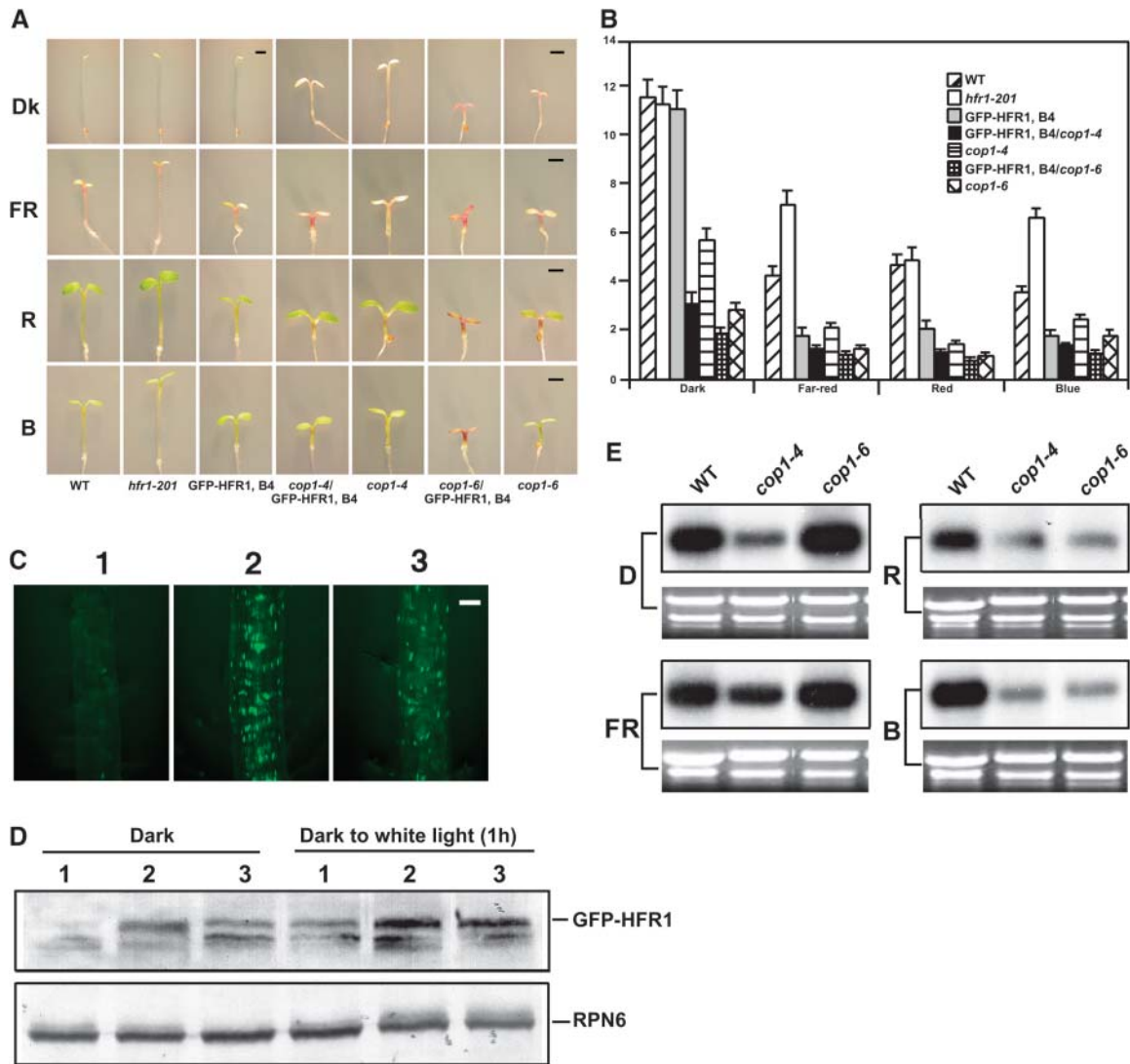


Figure 7. HFR1 Protein Degradation Is Defective in *cop1* Mutants.

(A) Seedling morphology showing that the *cop1* mutant phenotype is enhanced by the *GFP-HFR1* transgene under all light conditions. Photographs of seedlings from each light condition except darkness were taken at the same magnification. For dark-grown seedlings, the three seedlings on the left side were of the same magnification, whereas the four seedlings on the right side were of another magnification. Dk, darkness; FR, far red; R, red; B, blue. Bars = 1 mm.

(B) Quantification of hypocotyl lengths of various mutants under different light conditions. Bars stand for standard deviations.

(C) Fluorescence images of GFP-HFR1 in dark-grown root cells. Panel 1, parental *GFP-HFR1* transgenic seedlings, line B4; panels 2 and 3, *cop1-4* and *cop1-6* seedlings harboring the *GFP-HFR1* transgene, respectively. Bar = 50 μ m.

(D) Effects of *cop1* mutations on HFR1 protein accumulation in seedlings grown either in continuous darkness for 5 d or grown in darkness for 5 d then transferred to white light for 1 h. An immunoblot of anti-RPN6 is shown below to indicate approximately equal loadings. Lane 1, parental *GFP-HFR1* transgenic seedlings, line B4; lanes 2 and 3, *cop1-4* and *cop1-6* seedlings, respectively, harboring the *GFP-HFR1* transgene.

(E) Effects of *cop1* mutations on *HFR1* transcript accumulation under different light conditions. Seedlings were grown in darkness for 5 d or grown in darkness (D) for 4 d and then transferred to continuous far-red (FR), red (R), or blue light (B) for 24 h. In each blot, *HFR1* transcript is shown on the top and a UV fluorescence image of the rRNA is shown below as a loading control.

dark and far-red light. Next, effects of these *cop1* mutations on GFP-HFR1 protein accumulation were examined. Fluorescence microscopy examination revealed that root cells of *cop1-4* and *cop1-6* mutants accumulated significant amounts of GFP-HFR1 protein in the nucleus, whereas root cells of dark-grown parental

GFP-HFR1 transgenic plants had barely detectable GFP-HFR1 (Figure 7C). An immunoblot analysis confirmed that dark-grown *cop1-4* and *cop1-6* mutants accumulated GFP-HFR1, whereas the parental *GFP-HFR1* did not. In addition, higher levels of GFP-HFR1 accumulated in *cop1-4* and *cop1-6* seedlings grown in

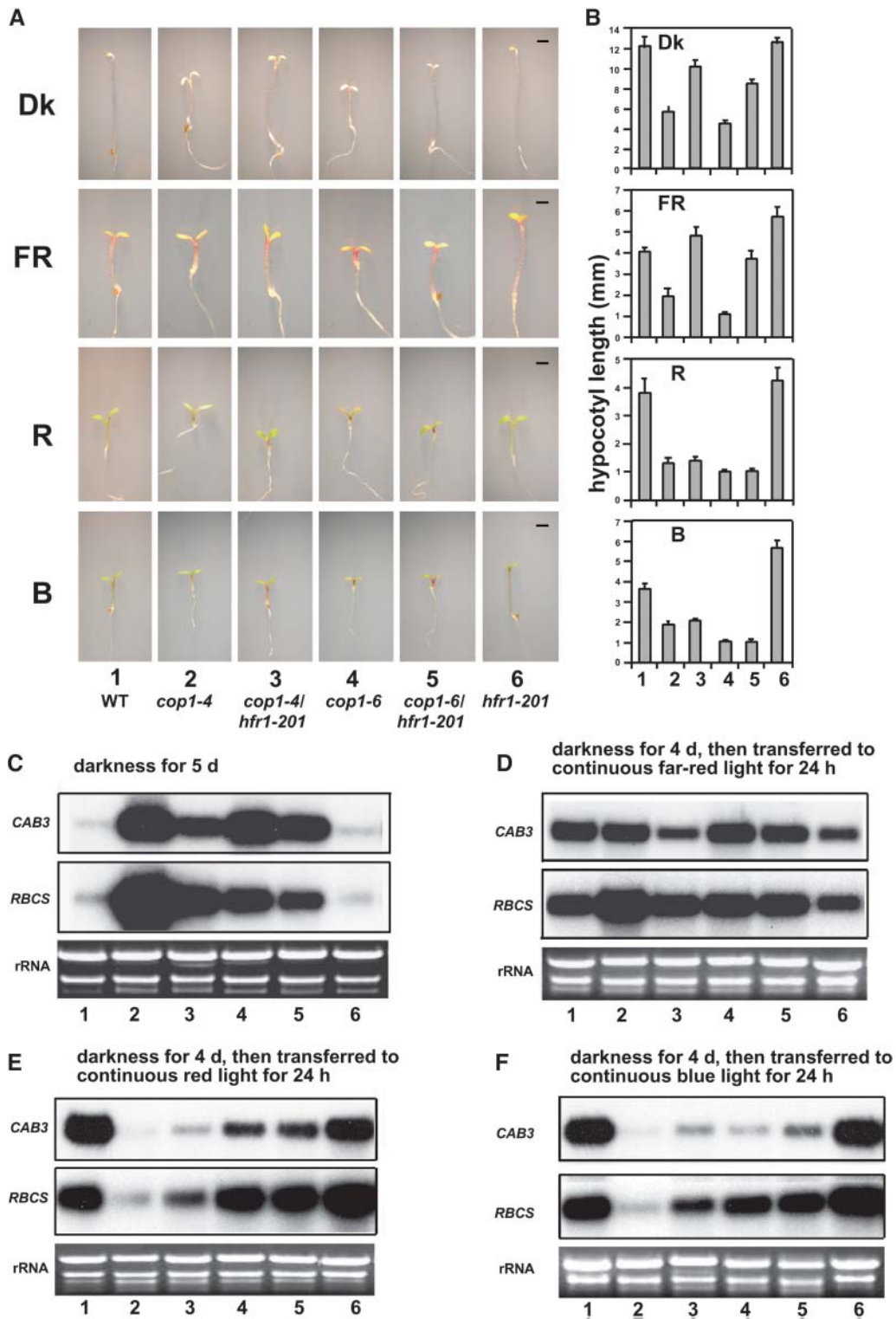


Figure 8. Epistasis Analyses of *hfr1-201* and *cop1* Mutations.

(A) Seedling morphology of seedlings grown under continuous darkness (Dk) and far-red (FR), red (R), and blue (B) light conditions. Photographs of seedlings from each light condition were taken at the same magnification. The genotypes of seedlings shown in all panels in this figure are as follows: 1, the wild type; 2, *cop1-4*; 3, *cop1-4 hfr1-201*; 4, *cop1-6*; 5, *cop1-6 hfr1-201*; 6, *hfr1-201*. All mutations are in the same ecotype background (*Columbia*). Bars = 1 mm.

darkness for 5 d, then transferred to white light for 1 h, compared with similarly treated parental GFP-HFR1 plants (Figure 7D). These results indicate that COP1 is required for the regulated degradation of HFR1 in both darkness and in the light and support a critical role of COP1 in desensitizing light signaling by acting as an E3 ligase for HFR1 in vivo.

We also examined the effects of *cop1* mutations on *HFR1* transcript accumulation under different light conditions. Slightly more *HFR1* transcript accumulated in the *cop1-6* mutant seedlings grown under continuous darkness for 5 d or *cop1-6* mutant seedlings grown under continuous darkness for 4 d and then transferred to far-red light for 24 h. However, less *HFR1* transcripts were detected in *cop1-4* under the above light conditions. Furthermore, compared with wild-type control plants, less *HFR1* transcripts accumulated in both *cop1-4* and *cop1-6* seedlings that were grown under continuous darkness for 4 d and then transferred to red or blue light for 24 h (Figure 7E), indicating that the effects of *cop1* mutations on *HFR1* transcript levels are allele and light quality dependent.

HFR1 Acts Downstream of COP1

A previous study employing the *cop1-6 hfr1-201* double mutant suggested that HFR1 acts downstream of COP1 and is necessary for a subset of *cop1*-mediated photomorphogenic phenotypes in the dark, including inhibition of hypocotyl elongation, gravitropic hypocotyl growth, and expression of the light-inducible genes *CAB* and *RBCS* (Kim et al., 2002). To determine whether such an epistatic relationship between *cop1* and *hfr1* is allelic specific, we constructed double mutants between *hfr1-201* and both *cop1-4* and *cop1-6* (all these mutations are in the *Columbia* background). In agreement with the previous observation (Kim et al., 2002), *hfr1-201* partially suppressed the constitutive photomorphogenic phenotype of *cop1-4* and *cop1-6* in darkness (although the *hfr1-201* mutant itself etiolated normally in darkness) and almost completely suppressed the hypersensitive phenotype of *cop1-4* and *cop1-6* under far-red light, supporting the proposition that HFR1 acts downstream of COP1. Interestingly, although the *hfr1-201* mutant exhibits reduced deetiolation under blue light, it failed to suppress the hypersensitivity of *cop1* mutants under blue light, nor did it suppress the hypersensitivity of *cop1* mutants under red light (Figures 8A and 8B).

To provide molecular evidence for the observed genetic interactions between *hfr1* and *cop1* mutations, we examined expression of several light-responsive genes in the *hfr1-201* and *cop1* mutants and their double mutants in various light conditions. As shown in Figure 8C, for seedlings grown under

continuous darkness for 5 d, the elevated expression of both *CAB3* and *RBCS* in *cop1-4* and *cop1-6* mutants was effectively suppressed by the *hfr1-201* mutation. For seedlings grown in continuous darkness for 4 d and then transferred to far-red light for 24 h, *CAB3* gene expression was comparable in wild-type plants, the *cop1-4*, and the *cop1-6* mutants but was reduced in the *hfr1-201* mutants. *CAB3* expression in the *cop1-4 hfr1-201* and *cop1-6 hfr1-201* double mutants was reduced compared with their *cop1* parental mutants. Compared with wild-type plants, *RBCS* expression was much higher in *cop1-4* and marginally higher in *cop1-6* mutants but was clearly lower in the *hfr1-201* mutants. The *cop1-4 hfr1-201* double mutants had reduced *RBCS* expression compared with the *cop1-4* single mutants, although the *cop1-6 hfr1-201* double mutants had a similar level of *RBCS* expression as the *cop1-6* single mutants (Figure 8D). Thus, these results support the notion that HFR1 acts downstream of COP1 under darkness and far-red light conditions. Most strikingly, for seedlings grown in continuous darkness for 4 d and then transferred to red or blue light for 24 h, expression of *CAB3* and *RBCS* was much lower in the *cop1-4* and *cop1-6* mutants compared with wild-type plants. *CAB3* expression was marginally affected in the *hfr1-201* mutants, but *RBCS* expression was clearly elevated in the *hfr1-201* mutants under these two light conditions. Both *cop1-4 hfr1-201* and *cop1-6 hfr1-201* double mutants had intermediate levels of *CAB3* and *RBCS* expression compared with their parental mutants (Figures 8E and 8F). These results indicate that COP1 and HFR1 regulation of light-responsive gene expression is light quality dependent and gene specific and possibly entails feedback regulation.

COP1-HFR1 Interaction Is Essential for COP1-Mediated Degradation of HFR1

To test whether the COP1-HFR1 interaction is required for the observed degradation of HFR1, we generated transgenic plants overexpressing a C-terminal 161-amino acid fragment of HFR1 fused with GFP at the N terminus (*GFP-CT161*). CT161 of HFR1 contains the bHLH DNA binding domain and the rest of the C-terminal portion but lacks the COP1-interacting N-terminal 131 amino acids. In both the wild-type and *hfr1-201* mutant backgrounds, the transgenic plants etiolated normally but displayed significantly enhanced photomorphogenesis under far-red, red, and blue light, similar to the effects of overexpressing GFP-tagged full-length *HFR1* (Figures 9A and 9B; data not shown). This result suggests that *GFP-CT161* is functionally autonomous in promoting light signaling and that the enhanced light signaling could be due to a defect in COP1-mediated

Figure 8. (continued).

(B) Quantification of hypocotyl lengths. Bars stand for standard deviations.

(C) *CAB3* and *RBCS* expression in dark-grown seedlings.

(D) *CAB3* and *RBCS* expression seedlings grown in darkness for 4 d and then transferred to far-red light for 24 h.

(E) *CAB3* and *RBCS* expression seedlings grown in darkness for 4 d and then transferred to red light for 24 h.

(F) *CAB3* and *RBCS* expression seedlings grown in darkness for 4 d and then transferred to blue light for 24 h. For **(C)** to **(F)**, a representative UV fluorescence image of rRNA from the duplicating gels of each light condition is shown as a loading control.

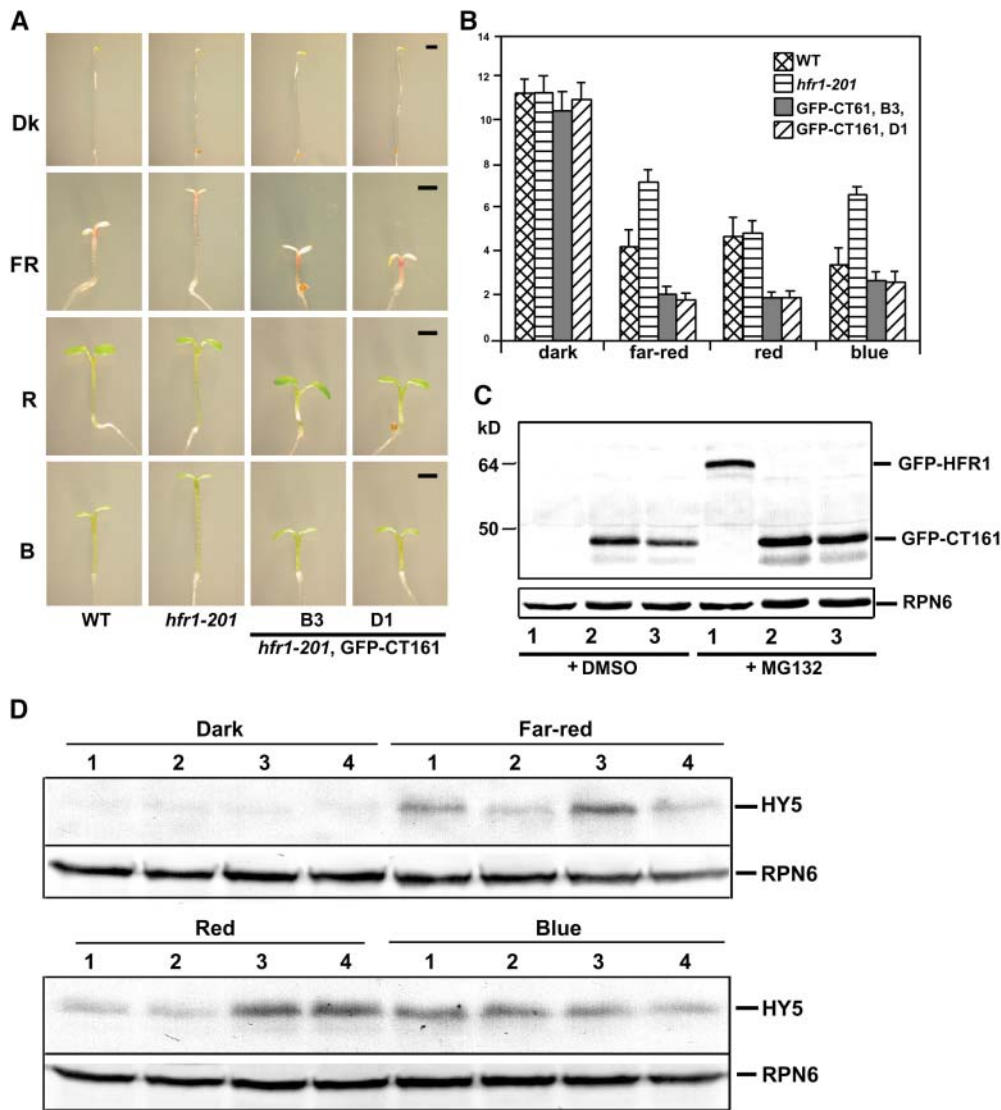


Figure 9. CT161 of HFR1 Is Functional Autonomous in Promoting Light Signaling, whereas NT131 Is Required for COP1-Mediated Destabilization of HFR1.

(A) Overexpression GFP-CT161 in the *hfr1-201* mutant background causes a strong hypersensitive response to far-red (FR), red (R), and blue light (B) and normal etiolation growth in the dark (Dk). Two independent lines are shown. Photographs of seedlings from each light condition were taken at the same magnification. WT, wild-type plants. Bars = 1 mm.

(B) Quantification of hypocotyl lengths under various light conditions. Bars stand for standard deviations.

(C) Immunoblot showing that GFP-CT161 accumulates in dark-grown seedlings, whereas no GFP-HFR1 was detected in the absence of MG132. MG132 treatment stabilizes GFP-HFR1 but has minimal effects on the accumulation of GFP-CT161. An immunoblot of anti-RPN6 was shown below to indicate approximately equal loadings. Lane 1, GFP-HFR1 transgenic line D3; lanes 2 and 3, GFP-CT161 transgenic lines B3 and D1, respectively.

(D) Effects of *hfr1-201* mutation, GFP-HFR1, and GFP-CT161 transgenes on HY5 accumulation. Seedlings were grown in continuous darkness, far-red, red, or blue light for 5 d, then total protein was extracted for immunoblot analysis. Immunoblots of anti-RPN6 are shown below to indicate approximately equal loadings. Lane 1, the wild type; lane 2, *hfr1-201* mutants; lane 3, GFP-HFR1 seedlings; lane 4, GFP-CT161 seedlings.

destabilization of HFR1. This observation is consistent with a recent report that overexpression of HFR1- Δ N105, which contains the C-terminal 188 amino acids of HFR1 (27 amino acids more than CT161), activated a branch pathway of light signaling that mediates a subset of photomorphogenic responses, including seed germination, deetiolation, gravitropic hypocotyls

growth, blocking of greening, and expression of several light-regulated genes, including *CAB*, *PSAE*, *PSBL*, *DRT112*, *PORA*, and *XTR7* (Yang et al., 2003). However, our GFP-CT161 transgenic plants did not display a constitutive photomorphogenic phenotype observed with the HFR1- Δ N105 transgenic plants (Yang et al., 2003). This constitutive photomorphogenic

phenotype was not detected with our *Myc-CT161* transgenic plants either, although this transgene, similar to *GFP-CT161*, caused enhanced photomorphogenesis in response to far-red, red, and blue light (data not shown). Thus, the discrepancy regarding the dark-grown phenotypes of our *GFP (Myc)-CT161* transgenic plants and the *HFR1-ΔN105* transgenic plants (Yang et al., 2003) is possibly due to the size difference in the transgenes used in these two independent studies. Alternatively, the Myc and GFP tags in our transgenes might affect the function of CT161 in dark-grown seedlings.

To confirm that the N-terminal COP1-interacting domain of HFR1 is required for COP1-mediated degradation of HFR1, we compared the protein levels of GFP-HFR1 and GFP-CT161 in dark-grown seedlings. As shown in Figure 9C, levels of GFP-CT161 were much higher than those of GFP-HFR1, and MG132 treatment effectively blocked the degradation of GFP-HFR1 in darkness, but it had minimal effects on the accumulation of GFP-CT161. RNA gel blot analysis showed that the transcripts levels of *GFP-HFR1* and *GFP-CT161* were comparable in dark-grown seedlings (data not shown). Thus, we concluded that the N-terminal COP1-interacting domain of HFR1 serves as a regulatory module of HFR1 stability and is required for COP1-mediated proteolysis of HFR1.

Previous studies have shown that another substrate protein targeted for degradation by COP1 in darkness, the basic domain/leucine zipper transcription factor HY5, acts in a separate pathway from HFR1 to promote photomorphogenesis (Osterlund et al., 2000; Kim et al., 2002). To further test the functional relationship of HY5 and HFR1 at the molecular level, we examined HY5 accumulation in the wild type, the *hfr1-201* mutant, and the *GFP-HFR1* and *GFP-CT161* transgenic seedlings under different light conditions. As shown in Figure 9D, all dark-grown seedlings had barely detectable levels of HY5. Under continuous far-red light, *hfr1-201* mutant seedlings had slightly reduced HY5 levels compared with wild-type plants. Interestingly, the *GFP-HFR1* transgenic seedlings, but not the *GFP-CT161* seedlings, had significantly higher levels of HY5. Under continuous red light, HY5 levels were comparable in wild-type and *hfr1-201* mutant seedlings but increased in both the *GFP-HFR1* and *GFP-CT161* seedlings. Under continuous blue light, HY5 levels were also comparable in wild-type and *hfr1-201* mutant seedlings but slightly reduced in both the *GFP-HFR1* and *GFP-CT161* seedlings. These results suggest that HFR1 might be involved in regulating *HY5* expression or *HY5* accumulation in a light quality-dependent manner. In addition, the observation that *HY5* was degraded normally in dark-grown *GFP-CT161* transgenic seedlings provided strong support that accumulation of GFP-CT161 in dark-grown seedlings is due to the lack of its N-terminal COP1-interacting domain rather than a general defect in COP1 activity conferred by the transgene.

DISCUSSION

Regulated Proteolysis of Key Regulatory Proteins in Light Signaling

In this study, we substantiate the view that HFR1 defines a key regulator of Arabidopsis seedling photomorphogenesis. Seed-

lings overexpressing *GFP-HFR1* displayed drastically enhanced photomorphogenesis under all light conditions, including far-red, red, and blue light, suggesting that HFR1 is necessary and sufficient for the activation of light signaling pathway, irrespective of light quality. We show that HFR1 is a short-lived protein in darkness and is rapidly degraded through a 26S proteasome-dependent manner, indicating that HFR1 is subject to regulated proteolysis.

Many other key light signaling factors have been previously demonstrated to be regulated via proteolysis, including phyA (Seo et al., 2004), cry2 (Lin et al., 1998), HY5 (Osterlund et al., 2000; Saijo et al., 2003), HYH (Holm et al., 2002), LAF1 (Seo et al., 2003), and PIF3 (Bauer et al., 2004; Park et al., 2004). Consistent with these findings, a group of photomorphogenesis-repressing COP/DET/FUS proteins encoded by 11 pleiotropic loci are all likely involved in regulated proteolysis (Wei and Deng, 1996, 2003; Serino and Deng, 2003; Sullivan et al., 2003). Among them, COP1 is a 76-kD RING finger protein with E3 ligase activity and is responsible for the ubiquitination and degradation of HY5, HYH, LAF1, and phyA (Osterlund et al., 2000; Holm et al., 2002; Saijo et al., 2003; Seo et al., 2003, 2004). DET1 was originally proposed to be involved in controlling chromatin remodeling and gene expression (Benvenuto et al., 2002; Schroeder et al., 2002). However, a recent study showed that a mammalian homolog of Arabidopsis DET1 is implicated in regulating ubiquitination of c-Jun together with a mammalian COP1 (Wertz et al., 2004). The COP9 signalosome (CSN) is a nuclear-enriched protein complex sharing similarity to the lid subcomplex of the 26S proteasome and acts to deconjugate NEDD8/Rub1 from the cullin subunit of SKP1/Cullin/F-box-type E3 ligase (Schwechheimer et al., 2001). It has been reported that CSN interacts with the 26S proteasome (Kwok et al., 1999; Peng et al., 2003). COP10 is a ubiquitin conjugating enzyme variant (Suzuki et al., 2002), which forms a complex with DDB1 and DET1. COP10 also physically interacts with COP1, CSN, and ubiquitin-conjugating enzymes (E2s) and can enhance E2 activity in vitro (Yanagawa et al., 2004). Thus, modulation of the intracellular levels of activated photoreceptors and other signaling molecules by regulated proteolysis is likely to be a central theme for controlling the specificity and magnitude of light signaling.

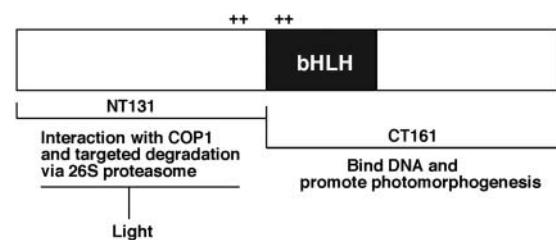


Figure 10. A Model Depicting the Structure-Function Relationship of HFR1.

The N-terminal 131-amino acid fragment (NT131) is involved in interaction with COP1 and is essential for COP1-mediated destabilization of HFR1. The C-terminal 161-amino acid fragment (CT161) is autonomous in DNA binding and promoting photomorphogenesis. Light abrogates COP1-mediated destabilization of HFR1 by depleting the nuclear abundance of COP1 (represented by a bar). ++, putative nuclear localization signals.

Critical Role of HFR1 during the Transition from Etiolated Growth to Photomorphogenesis

In this study, we show that HFR1 protein is completely degraded in <6 h when seedlings are transferred from red light to dark-grown conditions. This observation suggests that degradation of HFR1 might be a key regulatory mechanism of repressing photomorphogenesis in the dark. The high levels of *HFR1* transcript (Duek and Fankhauser, 2003) and low levels of HFR1 protein in darkness and rapid stabilization of HFR1 protein upon exposure to light likely provide the plants with a jump-start mechanism to initiate photomorphogenesis without undergoing the more time- and energy-consuming route of *de novo* transcription/translation. This feature may enable the plants to respond to changes in their light environment with minimal delay and might be vital for plants to maximize their chances of survival in their natural environment. In this regard, it is particularly interesting to point out that two HFR1-related bHLH proteins, phytochrome-interacting factor 1 (PIF1) and PIF3, also play important roles in preparing subterranean seedlings for the transition to photoautotrophic growth. PIF1 plays a critical role in regulating chlorophyll biosynthesis when seedlings emerge from subterranean darkness into sunlight. Dark-grown *pif1* mutant seedlings accumulate excess free protochlorophyllide, which causes lethal seedling bleaching upon exposure to light (Huq et al., 2004). PIF3 acts mainly as a negative regulator of phyB-mediated red light inhibition of hypocotyl elongation and cotyledon expansion (Kim et al., 2003). PIF3 protein is rapidly degraded in red and far-red light and it readily reaccumulates to high levels in the dark (Bauer et al., 2004; Park et al., 2004). These results suggest that PIF3 might be mainly required for phytochrome signaling during the developmental transition from etiolated growth to photomorphogenesis or vice versa (Bauer et al., 2004). Together, accumulating data suggest that these bHLH transcription factors are important regulators of the switch between skotomorphogenic and photomorphogenic developmental programs for Arabidopsis seedlings.

COP1 Is an E3 Ligase Responsible for Targeted Degradation of HFR1

In this study, we demonstrate that HFR1 physically interacts with COP1, an E3 ubiquitin ligase, and show that COP1 exhibits ubiquitin ligase activity toward HFR1 *in vitro*. HFR1 accumulation is higher in *cop1* reduction-of-function mutants, suggesting that COP1 is required for targeted degradation of HFR1 *in vivo*. Genetic epistasis analyses suggested that HFR1 acts downstream of COP1. Moreover, plants overexpressing a C-terminal 161-amino acid fragment of HFR1 (CT161) display exaggerated photomorphogenesis, suggesting that CT161 of HFR1 is functionally autonomous in binding DNA and promoting light signaling. Furthermore, this truncated HFR1 gene product is more stable than the full-length HFR1 protein in darkness, indicating that the COP1-interacting N-terminal portion of HFR1 serves as a regulatory module and is essential for COP1-mediated destabilization of HFR1 (Figure 10).

Currently, it is not clear yet how light abrogates targeted degradation of HFR1 by COP1. Because COP1 displays a light-

mediated nucleocytoplasmic repartitioning, being enriched in the nucleus in the dark and depleted from the nucleus in the light (von Arnim and Deng, 1994), one possibility is that light reduces the nuclear abundance of COP1. Our observation that light, irrespective of light quality, promotes HFR1 protein accumulation is consistent with the report that far-red, red, blue, and white light are all capable of reducing COP1 nuclear abundance (Osterlund and Deng, 1998).

It should be noted that the slow kinetics of COP1 nuclear depletion (could take up to 24 h; von Arnim and Deng, 1994) and the observed rapid degradation of HFR1 and HY5 (Osterlund et al., 2000) suggest that at least one additional unknown regulatory event is required to account for the early and rapid inactivation of COP1 by light. Blue light signaling has been reported to involve a direct protein interaction of cryptochromes with COP1 (Wang et al., 2001; Yang et al., 2001). The red light photoreceptor phyB has also been reported to interact with COP1 in a yeast two-hybrid assay (Yang et al., 2001). However, the authenticity of such an interaction and its physiological relevance remain to be established. In addition, it was shown that COP1 can physically interact with the far-red photoreceptor phyA and acts as an E3 ligase for phyA, leading to its elimination and subsequent termination of phyA signaling (Seo et al., 2004). Possible effects of activated phyA on COP1 activity have not been addressed. It is possible that, before its degradation, the activated phyA could inactivate COP1 through unknown mechanisms. Moreover, SPA1, a nuclear-localized repressor of far-red light signaling (Hoecker et al., 1998, 1999), and three additional SPA1-related proteins, named SPA2, SPA3, and SPA4, may also contribute to the regulation of COP1 activity through direct protein-protein interaction with COP1 to fine-tune light signaling (Laubinger and Hoecker, 2003; Laubinger et al., 2004). Further studies are required to determine the effects of different light qualities and how other factors may contribute to the regulation of HFR1 stability and activity in light signaling.

METHODS

Plant Materials and Growth Conditions

The wild type, various mutants, and transgenic plants were of the *Columbia* ecotype. The *cop1-4* and *cop1-6* mutants have been described (McNellis et al., 1994). Seeds were sterilized by incubation in freshly prepared 30% bleach plus 0.01% (v/v) Triton X-100 for 15 min and then washed four times with sterile water. The surface-sterilized seeds were sown on MS germination plates (1 × MS media supplied with 1% sucrose) and were cold treated for 3 d at 4°C. After exposure to white light for 24 h to stimulate germination, plates with seeds were transferred to appropriate light conditions for 4 to 5 d at 22°C. Far-red, red, and blue light was supplied by LED light sources, with irradiance fluence rates of ~0.5 μmol/m²s, 30 μmol/m²s, and 5 μmol/m²s, respectively, unless otherwise indicated (measured with International Light model IL1400A with sensor model SEL-033/F/W; Newburyport, MA). White light was supplied by cool-white fluorescent lamps.

For MG132 and DMSO treatments, seedlings were first grown on germination plates to desired stages, then they were collected by forceps and incubated in liquid MS medium containing MG132 (50 μM, dissolved in DMSO) or 0.1% DMSO under light conditions as indicated in the text. After the incubation, the seedlings were thoroughly washed with liquid

MS medium three times (5 min each) to remove residual DMSO or MG132 before proceeding with the experimental procedure.

Plasmid Construction

A full-length HFR1 cDNA fragment was generated by RT-PCR using the primer pair HFR1U (5'-AGGATCCCCATGTGCGAATAATCAAGCTTTCATGG-3') and HFR1D (5'-ACTCGAGTCATAGTCTTCTCATCGCATGG-3'), and the PCR product was cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) to generate the pTOPO-HFR1 clone. Various domain deletion HFR1 mutants were generated by PCR with pTOPO-HFR1 as the template. To facilitate subcloning, proper restriction sites were incorporated into the 5' ends of the primers. Primer pairs used to produce various deletion derivatives of HFR1 were as follows: HFR1-NT189, HFR1-1F (5'-GAGAGATCTGAATTCATGTGCGAATAATCAAGCTTTC-3') and HFR1-189R (5'-GGCTCTAGACTCGATGCATCTGAAGTTGAAGTTGAAG-3'); HFR1-NT131, HFR1-1F and HFR1-131R (5'-GGCTCTAGACTCGAGT-CATCTTGTAACTCCTCCGATTC-3'); HFR1-CT161, HFR1-132F (5'-GAGAGATCTGAATTCATGGAAGTTCCTTCAGTTACTCG-3') and HFR1-292R (5'-GGCTCTAGACTCGAGTCATAGTCTTCTCATCGCATGGAAAG-3'); HFR1-CT106, HFR1-187F (5'-GAGAGATCTGAATTCATGCTTCAGATGATGTCAACAGTG-3') and HFR1-292R; HFR1-HLH, HFR1-132F, and HFR1-189R. To generate HFR1- Δ HLH, the N-terminal and C-terminal portions of HFR1 were obtained with two separate PCRs using primer pairs HFR1-1F with HFR1- Δ HLHR (5'-CACTGTTGACATCATCTGAAGTCTTGTAACTCCTCCGATTC-3') and HFR1- Δ HLHF (5'-GAATCGGAGGAGTTTACAAGACTTCAGATGATGTCAACAGTG-3') with HFR1-292R. Then a mixture of their PCR products was used as the template for a third PCR with primer pair HFR1-1F and HFR1-292R. These PCR products were cloned into pCR-TOPO2.1 vector to produce their respective pTOPO derivative clones. All PCR inserts were validated by DNA sequencing.

To generate *Myc-HFR1* transgenic plants, the pTOPO-HFR1 clone was digested with *EcoRI* (blunted with Klenow enzyme) and *XhoI*, and the released HFR1 insert was then cloned into the *PstI* (blunted with Klenow enzyme) and *XhoI* sites of an intermediate vector named pMyc-5' to generate pMyc-HFR1 in which nine copies of Myc epitope tag were fused to the 5' end of the HFR1 opening reading frame. A *SpeI-XhoI* fragment containing the 35S promoter, Myc-tagged HFR1, and the Nos terminator sequence was released and cloned into the *XbaI* and *XhoI* sites of an in-house generated binary vector pJIM19 (J. Sullivan, unpublished data) to produce pJIM-Myc-HFR1.

To generate *GFP-HFR1* and *GFP-CT161* transgenic plants, a *BamHI* (blunted with Klenow enzyme)-*SpeI* fragment containing the full-length HFR1 cDNA was released from the pTOPO-HFR1 clone and ligated into the *BglII* (blunted with Klenow enzyme) and *XbaI* sites of pRTL2-mGFP (S65T) vector (von Arnim et al., 1998) to produce the pRTL2-mGFP-HFR1 clone. A *BglII-XbaI* fragment of HFR1-CT161 was released from pTOPO-CT161 and cloned into the corresponding sites of the pRTL2-mGFP (S65T) vector to form pRTL2-mGFP-CT161. Then, *PstI* fragments containing the 35S promoter, the GFP-HFR1 or GFP-CT161 fusion gene, and the 35S terminator were released from pRTL2-mGFP-HFR1 and pRTL2-mGFP-CT161 and cloned into the *PstI* site of the binary vector pPZP221 (Hajdukiewicz et al., 1994), resulting in pPZP221-GFP-HFR1 and pPZP221-GFP-CT161, respectively.

To generate constructs for yeast two-hybrid assay, full-length HFR1 or its various deletion derivatives were released as *EcoRI-XhoI* fragments from their respective pTOPO clones and ligated into the corresponding sites of the vector pEG202 (Wang et al., 2001) to produce translational fusions with the LexA DNA binding domain.

To express GST-NT131 recombinant protein in *Escherichia coli*, an *EcoRI-XhoI* fragment containing HFR-NT131 was released from pRTL2mGFP-NT131 and cloned into the expression vector pGEX5-1 to generate pGEX-NT131.

To generate GAD-HFR1, an *NcoI-XhoI* PCR fragment of full-length HFR1 was cloned into the corresponding sites of the vector pACT2 (Clontech, Palo Alto, CA).

Plant Transformation and Selection of Transgenic Plants

The pJIM-Myc-HFR1, pPZP221-GFP-HFR1, and pPZP221-GFP-CT161 binary constructs were electroporated into the *Agrobacterium tumefaciens* strain GV3101 and then introduced into *Arabidopsis thaliana* wild-type plants and the *hfr1-201* mutants via a floral dip method (Clough and Bent, 1998). Transgenic plants were selected on germination plates containing 20 μ g/mL of glufosinate-ammonium (for *Myc-HFR1*) or 100 μ g/mL of gentamycin (for *GFP-HFR1* or *GFP-CT161*). We selected ~40 T1 transgenic lines with single T-DNA insertions and allowed them to self to produce T2 seeds. Phenotypic analyses were conducted with T2 plants and then confirmed in T3 generation. For most experiments, homozygous T3 or T4 transgenic plants were used.

Antibody Preparation and Immunoblot Analysis

An *EcoRI-XhoI* full-length HFR1 fragment was released from pTOPO-HFR1 and cloned into the pET28c vector (Novagen, Madison, WI), expressed and purified from *E. coli*, and used to raise polyclonal antibodies in rabbits. For immunoblots with Arabidopsis plant extracts, proteins were extracted with the following buffer: 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 25 mM β -glycerophosphate, 2 mM sodium orthovanadate, 10% glycerol, 0.1% Tween 20, 1 mM DTT, 1 mM PMSF, and 1 \times Complete protease inhibitor cocktail (Roche, Indianapolis, IN). Myc-HFR1 was detected with anti-myc monoclonal antibody (the 9E10 clone; Santa Cruz Biotechnology, Santa Cruz, CA), whereas GFP-HFR1 and GFP-CT161 were detected with rabbit anti-GFP polyclonal antibodies (Molecular Probes, Eugene, OR). HY5 was detected with anti-HY5 specific antibodies (Osterlund et al., 2000). The proteins were visualized by incubating with goat-anti-mouse or goat-anti-rabbit secondary antibodies conjugated with alkaline phosphatase in the presence of 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium as substrates.

Construction of Double Mutants

All double mutant combinations were derived from genetic crosses of their respective two single parental mutants (or transgenic lines). Putative double mutants were selected in F2 generation and confirmed in F3 generation based on the mutant phenotype and/or antibiotic selection markers.

RNA Gel Blotting

For RNA gel blotting, Arabidopsis seedlings were grown in different light conditions as indicated in the text. Total RNA was extracted using RNeasy plant mini kits (Qiagen, Valencia, CA). Approximately 5 μ g of RNA per lane was size fractionated on a formaldehyde agarose gel and subsequently transferred to a nylon membrane. After hybridization in 0.25 M sodium phosphate, 1 mM EDTA, 1% casein, and 7% SDS at 65°C with random prime-labeled fragments (Roche), membranes were washed two times each of 2 \times SSC, 0.1% SDS, 0.2 \times SSC, 0.1% SDS, and 0.1 \times SSC, 0.1% SDS. A full-length cDNA fragment of *HFR1* derived from pTOPO-HFR1 was used for probe labeling. Fragments of *CHS*, *CAB3*, and *RBCS* used for probe labeling were described previously (Wang and Deng, 2002).

Fluorescence Microscopy

To visualize the GFP-HFR1 and GFP-CT161 fusion proteins, transgenic seedlings expressing *GFP-HFR1* and *GFP-CT161* were mounted on

slides and examined with an Axioskop fluorescence microscope (Zeiss, Oberkochen, Germany) with GFP filter sets. Representative images were documented by photography with a digital Axiocam camera system (Zeiss). All images were taken from the same region of hypocotyls with identical exposure.

Transient Expression in Onion Epidermal Cells

The procedure and pRTL2-mGFP (S65T)-COP1 construct for transient expression in living onion epidermal cells using particle bombardment have been described (Ang et al., 1998).

In Vitro Interaction Assay

The procedure and constructs for the in vitro expression of GAD, COP1, and COP1- Δ CC were described by Hoecker and Quail (2001).

Yeast Two-Hybrid Analysis

The assay system and all the procedures have been described by Serino et al. (1999). The AD-COP1 and various AD fusions of COP1 derivatives were described previously (Ang et al., 1998; Holm et al., 2001).

In Vitro Ubiquitination Assays

GST-tagged HFR1 N-terminal 131-amino acid recombinant fusion protein (GST-NT131) was expressed in BL21 codon plus (Stratagene, La Jolla, CA) *E. coli* cells and affinity purified using glutathione matrix (Amersham, Buckinghamshire, UK). In vitro ubiquitination assays were performed in a total volume of 30 μ L consisting of 50 mM Tris, pH 7.5, 10 mM MgCl₂, 10 mM creatine phosphate, 10 mM ATP, 2 mg BSA, 1 unit creatine phosphokinase, 0.5 mg biotinylated ubiquitin (Affiniti, Exeter, UK), 5 ng rabbit E1 (Boston Biochem, Boston, MA), 10 ng rice (*Oryza sativa*) Rad6 (Yanagawa et al., 2004) as the E2, 500 ng MBP-COP1, 200 ng of GST-NT131, and 1 μ g biotinylated ubiquitin (Boston Biochem) supplemented with 10 μ g unlabeled ubiquitin (Sigma-Aldrich, St. Louis, MO). Reactions were incubated at 30°C for 2 h before being terminated by the addition of SDS sample buffer. Products conjugated with biotinylated ubiquitin were detected by incubation of immunoblots with streptavidin-conjugated horseradish peroxidase for biotinylated ubiquitin (Amersham), followed by chemiluminescent visualization.

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REFERENCES

- Ahmad, M., Jarillo, J.A., Smirnova, O., and Cashmore, A.R. (1998). The CRY1 blue light photoreceptor of *Arabidopsis* interacts with phytochrome A *in vitro*. *Mol. Cell* **1**, 939–948.

- Ang, L.-H., Chattopadhyay, S., Wei, N., Oyama, T., Okada, K., Batschauer, A., and Deng, X.W. (1998). Molecular interaction between COP1 and HY5 defines a regulatory switch for light control of *Arabidopsis* development. *Mol. Cell* **1**, 213–222.
- Bauer, D., Viczián, A., Kircher, S., Nobis, T., Nitschke, R., Kunkel, T., Panigrahi, K.C.S., Ádám, E., Fejes, E., Schäfer, E., and Nagy, F. (2004). Constitutive photomorphogenesis 1 and multiple photoreceptors control degradation of phytochrome interacting factor 3, a transcription factor required for light signaling in *Arabidopsis*. *Plant Cell* **16**, 1433–1445.
- Benvenuto, G., Formiggini, F., Laflamme, P., Malakhov, M., and Bowler, C. (2002). The photomorphogenesis regulator DET1 binds the amino-terminal tail of histone H2B in a nucleosome context. *Curr. Biol.* **12**, 1529–1534.
- Briggs, W.R., and Olney, M.A. (2001). Photoreceptors in plant photomorphogenesis to date: Five phytochromes, two cryptochromes, one phototropin, and one superchrome. *Plant Physiol.* **125**, 85–88.
- Clough, S.J., and Bent, A.F. (1998). Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743.
- Deng, X.W., Matsui, M., Wei, N., Wagner, D., Chu, A.M., Feldmann, K.A., and Quail, P.H. (1992). *COP1*, an *Arabidopsis* regulatory gene, encodes a protein with both a zinc-binding motif and a G_B homologous domain. *Cell* **71**, 791–801.
- Deng, X.W., and Quail, P.H. (1999). Signaling in light-controlled development. *Semin. Cell Dev. Biol.* **10**, 121–129.
- Duek, P.D., and Fankhauser, C. (2003). HFR1, a putative bHLH transcription factor, mediates both phytochrome A and cryptochrome signaling. *Plant J.* **34**, 827–836.
- Fairchild, C.D., Schumaker, M.A., and Quail, P.H. (2000). *HFR1* encodes an atypical bHLH protein that acts in phytochrome A signal transduction. *Genes Dev.* **14**, 2377–2391.
- Fankhauser, C., and Chory, J. (2000). *RSF1*, an *Arabidopsis* locus implicated in phytochrome A signaling. *Plant Physiol.* **124**, 39–45.
- Hajdukiewicz, P., Svab, Z., and Maliga, P. (1994). The small, versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. *Plant Mol. Biol.* **25**, 989–994.
- Hoecker, U., and Quail, P.H. (2001). The phytochrome A-specific signaling intermediate SPA1 interacts directly with COP1, a constitutive repressor of light signaling in *Arabidopsis*. *J. Biol. Chem.* **276**, 38173–38178.
- Hoecker, U., Tepperman, J.M., and Quail, P.H. (1999). SPA1, a WD-repeat protein specific to phytochrome A signal transduction. *Science* **284**, 496–499.
- Hoecker, U., Xu, Y., and Quail, P.H. (1998). *SPA1*: A new genetic locus involved in phytochrome A-specific signal transduction. *Plant Cell* **10**, 19–33.
- Holm, M., Hardtke, C.S., Gaudet, R., and Deng, X.W. (2001). Identification of a structural motif that confers specific interaction with the WD40 repeat domain of *Arabidopsis* COP1. *EMBO J.* **20**, 118–127.
- Holm, M., Ma, L.-G., Qu, L.-J., and Deng, X.W. (2002). Two interacting bZIP proteins are direct targets of COP1-mediated control of light-dependent gene expression in *Arabidopsis*. *Genes Dev.* **16**, 1247–1259.
- Huq, E., Al-Sady, B., Hudson, M., Kim, C., Apel, K., and Quail, P.H. (2004). Phytochrome-interacting factor 1 is a critical bHLH regulator of chlorophyll biosynthesis. *Science* **305**, 1937–1941.
- Kendrick, R.E., and Kronenberg, G.H.M. (1994). *Photomorphogenesis in Plants*. (Dordrecht, The Netherlands: Kluwer Academic Publishers).
- Kim, J., Yi, H., Choi, G., Shin, B., Song, P.-S., and Choi, G. (2003). Functional characterization of phytochrome interacting factor 3 in phytochrome-mediated light signal transduction. *Plant Cell* **15**, 2399–2407.

- Kim, Y.-M., Woo, J.-C., Song, P.-S., and Soh, M.-S. (2002). HFR1, a phytochrome A-signaling component, acts in a separate pathway from HY5, downstream of COP1 in *Arabidopsis thaliana*. *Plant J.* **30**, 711–719.
- Kwok, S.F., Staub, J.M., and Deng, X.W. (1999). Characterization of two subunits of *Arabidopsis* 19S proteasome regulatory complex and its possible interaction with the COP9 complex. *J. Mol. Biol.* **285**, 85–95.
- Laubinger, S., Fittinghoff, K., and Hoecker, U. (2004). The SPA quartet: A family of WD-repeat proteins with a central role in suppression of photomorphogenesis in *Arabidopsis*. *Plant Cell* **16**, 2293–2306.
- Laubinger, S., and Hoecker, U. (2003). The SPA1-like proteins SPA3 and SPA4 repress photomorphogenesis in the light. *Plant J.* **35**, 373–385.
- Lin, C. (2002). Blue light receptors and signal transduction. *Plant Cell* **14** (suppl.), S207–S225.
- Lin, C., Yang, H., Guo, H., Mockler, T., Chen, J., and Cashmore, A.R. (1998). Enhancement of blue-light sensitivity of *Arabidopsis* seedlings by a blue light receptor cryptochrome 2. *Proc. Natl. Acad. Sci. USA* **95**, 2686–2690.
- McNellis, T.W., and Deng, X.W. (1995). Light control of seedling morphogenetic pattern. *Plant Cell* **7**, 1749–1761.
- McNellis, T.W., Von Arnim, A.G., Araki, T., Komeda, Y., Misera, S., and Deng, X.W. (1994). Genetic and molecular analysis of an allelic series of *cop1* mutants suggests functional roles for the multiple protein domains. *Plant Cell* **6**, 487–500.
- Nagatani, A., Reed, J.W., and Chory, J. (1993). Isolation and initial characterization of *Arabidopsis* mutants that are deficient in phytochrome A. *Plant Physiol.* **102**, 269–277.
- Neff, M.M., Fankhauser, C., and Chory, J. (2000). Light: An indicator of time and place. *Genes Dev.* **14**, 257–271.
- Osterlund, M.T., and Deng, X.W. (1998). Multiple photoreceptors mediate the light-induced reduction of GUS-COP1 from *Arabidopsis* hypocotyls nuclei. *Plant J.* **16**, 201–208.
- Osterlund, M.T., Hardtke, C., Wei, N., and Deng, X.W. (2000). Targeted destabilization of HY5 during light regulated development of *Arabidopsis*. *Nature* **405**, 462–466.
- Oyama, T., Shimura, Y., and Okada, K. (1997). The *Arabidopsis* HY5 gene encodes a bZIP protein that regulates stimulus-induced development of root and hypocotyl. *Genes Dev.* **11**, 2983–2995.
- Park, E., Kim, J., Lee, Y., Shin, J., Oh, E., Chung, W., Liu, J.R., and Choi, G. (2004). Degradation of phytochrome interacting factor 3 in phytochrome-mediated light signaling. *Plant Cell Physiol.* **136**, 968–975.
- Peng, Z., Shen, Y., Feng, S., Wang, X., Chitteti, B.N., Vierstra, R.D., and Deng, X.W. (2003). Evidence for a physical association of the COP9 signalosome, the proteasome, and specific SCF E3 ligases in vivo. *Curr. Biol.* **13**, R504–R505.
- Quail, P.H. (2002). Phytochrome photosensory signalling networks. *Nat. Rev. Mol. Cell Biol.* **3**, 85–93.
- Saijo, Y., Sullivan, J.A., Wang, H., Yang, J., Shen, Y., Rubio, V., Ma, L., Hoecker, U., and Deng, X.W. (2003). The COP1–SPA1 interaction defines a critical step in phytochrome A-mediated regulation of HY5 activity. *Genes Dev.* **17**, 2642–2647.
- Schroeder, D.F., Gahrtz, M., Maxwell, B.B., Cook, R.K., Kan, J.M., Alonso, J.M., Ecker, J.R., and Chory, J. (2002). De-etiolated 1 and damaged DNA binding protein 1 interact to regulate *Arabidopsis* photomorphogenesis. *Curr. Biol.* **12**, 1462–1472.
- Schwechheimer, C., Serino, G., Callis, J., Crosby, W.L., Lyapina, S., Deshaies, R.J., Gray, W.M., Estelle, M., and Deng, X.W. (2001). Interactions of the COP9 signalosome with the E3 ubiquitin ligase SCF^{TRIM} in mediating auxin response. *Science* **292**, 1379–1382.
- Seo, H.S., Watanabe, E., Tokutomi, S., Nagatani, A., and Chua, N.H. (2004). Photoreceptor ubiquitination by COP1 E3 ligase desensitizes phytochrome A signaling. *Genes Dev.* **18**, 617–622.
- Seo, H.S., Yang, J.-Y., Ishikawa, M., Bolle, C., Ballesteros, M., and Chua, N.-H. (2003). LAF1 ubiquitination by COP1 controls photomorphogenesis and is stimulated by SPA1. *Nature* **423**, 995–999.
- Serino, G., and Deng, X.W. (2003). The COP9 signalosome: Regulating plant development through the control of proteolysis. *Annu. Rev. Plant Biol.* **54**, 165–182.
- Serino, G., Tsuge, T., Kwok, S., Matsui, M., Wei, N., and Deng, X.W. (1999). *Arabidopsis cop8* and *fus4* mutations define the same gene that encodes subunit 4 of the COP9 signalosome. *Plant Cell* **11**, 1967–1980.
- Soh, M.S., Kim, Y.M., Han, S.J., and Song, P.S. (2000). REP1, a basic helix-loop-helix protein, is required for a branch pathway of phytochrome A signaling in *Arabidopsis*. *Plant Cell* **12**, 2061–2074.
- Sullivan, J.A., Shirasu, K., and Deng, X.W. (2003). The diverse roles of ubiquitin and the 26S proteasome in the life of plants. *Nat. Rev. Genet.* **4**, 948–958.
- Suzuki, G., Yanagawa, Y., Kwok, S.F., Matsui, M., and Deng, X.W. (2002). *Arabidopsis* COP10 is a ubiquitin-conjugating enzyme variant that acts together with COP1 and the COP9 signalosome in repressing photomorphogenesis. *Genes Dev.* **16**, 554–559.
- von Arnim, A.G., and Deng, X.W. (1994). Light inactivation of *Arabidopsis* photomorphogenic repressor COP1 involves a cell-specific regulation of its nucleocytoplasmic partitioning. *Cell* **79**, 1035–1045.
- von Arnim, A.G., Deng, X.W., and Stacey, M.G. (1998). Cloning vectors for the expression of green fluorescence protein fusion proteins in transgenic plants. *Gene* **221**, 35–43.
- Wang, H., and Deng, X.W. (2002). *Arabidopsis* FHY3 defines a key phytochrome A signaling component directly interacting with its homologous partner FAR1. *EMBO J.* **21**, 1339–1349.
- Wang, H., and Deng, X.W. (2003). Dissecting phytochrome A dependent signaling network in higher plants. *Trends Plant Sci.* **8**, 172–178.
- Wang, H., and Deng, X.W. (2004). Phytochrome signaling mechanisms. In *The Arabidopsis Book*, C.R. Somerville and E.M. Meyerowitz, eds (Rockville, MD: American Society of Plant Physiologists), doi/10.1199/tab.0016, <http://www.aspb.org/publications/arabidopsis/>.
- Wang, H., Ma, L.-G., Li, J.-M., Zhao, H.-Y., and Deng, X.W. (2001). Direct interaction of *Arabidopsis* cryptochromes with COP1 in light control development. *Science* **294**, 154–158.
- Wei, N., and Deng, X.W. (1996). The role of the *COP1/DET1/FUS* genes in light control of *Arabidopsis* seedling development. *Plant Physiol.* **112**, 871–878.
- Wei, N., and Deng, X.W. (2003). The COP9 signalosome. *Annu. Rev. Cell Dev. Biol.* **19**, 261–286.
- Wertz, I.E., O'Rourke, K.M., Zhang, Z., Dorman, D., Arnott, D., Deshaies, R.J., and Dixit, V.M. (2004). Human de-etiolated-1 regulates c-Jun by assembling a CUL4A ubiquitin ligase. *Science* **303**, 1371–1374.
- Whitelam, G.C., Johnson, E., Peng, J., Carol, P., Anderson, M.L., Cowl, J.S., and Harberd, N.P. (1993). Phytochrome A null mutants of *Arabidopsis* display a wild-type phenotype in white light. *Plant Cell* **5**, 757–768.
- Yanagawa, Y., Sullivan, J.A., Komatsu, S., Gusmaroli, G., Suzuki, G., Yin, J., Ishibashi, T., Saijo, Y., Rubio, V., Kimura, S., Wang, J., and Deng, X.W. (2004). *Arabidopsis* COP10 forms a complex with DDB1 and DET1 in vivo and enhances the activity of ubiquitin conjugating enzymes. *Genes Dev.* **18**, 2172–2181.
- Yang, H.Q., Tang, R.H., and Cashmore, A.R. (2001). The signaling mechanism of *Arabidopsis* CRY1 involves direct interaction with COP1. *Plant Cell* **13**, 2573–2587.
- Yang, K.-Y., Kim, Y.-M., Lee, S., Song, P.-S., and Soh, M.-S. (2003). Overexpression of a mutant basic helix-loop-helix protein HFR1, HFR1-ΔN105, activates a branch pathway of light signaling in *Arabidopsis*. *Plant Physiol.* **133**, 1630–1642.