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# The Degradation of HFR1, a Putative bHLH Class Transcription Factor Involved in Light Signaling, Is Regulated by Phosphorylation and Requires COP1

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## Summary

**All developmental transitions throughout the life cycle of a plant are influenced by light. In *Arabidopsis*, multiple photoreceptors including the UV-A/blue-sensing cryptochromes (cry1-2) and the red/far-red responsive phytochromes (phyA-E) monitor the ambient light conditions [1, 2]. Light-regulated protein stability is a major control point of photomorphogenesis [3]. The ubiquitin E3 ligase COP1 (constitutively photomorphogenic 1) regulates the stability of several light-signaling components [4–6]. HFR1 (long hypocotyl in far-red light) is a putative transcription factor with a bHLH domain acting downstream of both phyA and the cryptochromes [7–9]. HFR1 is closely related to PIF1, PIF3, and PIF4 (phytochrome interacting factor 1, 3 and 4), but in contrast to the latter three, there is no evidence for a direct interaction between HFR1 and the phytochromes [7, 10–12]. Here, we show that the protein abundance of HFR1 is tightly controlled by light. HFR1 is an unstable phosphoprotein, particularly in the dark. The proteasome and COP1 are required in vivo to degrade phosphorylated HFR1. In addition, HFR1 can interact with COP1, consistent with the idea of COP1 directly mediating HFR1 degradation. We identify a domain, conserved among several bHLH class proteins involved in light signaling [13, 14], as a determinant of HFR1 stability. Our physiological experiments indicate that the control of HFR1 protein abundance is important for a normal de-etiolation response.**

## Results and Discussion

### HFR1 Protein Accumulation Is Regulated by Light

It has been previously shown that during the first hours of de-etiolation, all light qualities are equally efficient to moderately induce *HFR1* mRNA levels [7–9, 15]. In order to better understand how HFR1 is regulated, we analyzed protein accumulation during de-etiolation. We prepared transgenic lines carrying an ectopic copy of the *HFR1* gene comprising the *HFR1* promoter, all introns, exons, and a triple HA tag inserted just after the last codon (referred to as HFR1 midi-gene, see Experimental Procedures). This construct complemented the *hfr1* mutant phenotype (Figure S1 in the Supplemental Data available with this article online) and allowed us to detect HFR1 by using an anti-HA antibody (Figure S2). In etiolated seedlings, HFR1 levels were low, and the protein accumulated to higher levels upon light exposure (Figure 1). A time course analysis showed that the highest HFR1 levels were reached around 2 hr after exposure to the light and that they decreased afterwards (Figures 1A and S3). Exposure to light also induced the appearance of a more slowly migrating band.

When the *HFR1* cDNA was expressed under the control of the constitutive cauliflower mosaic 35S promoter (35S:HFR1-HA), HFR1 protein levels were still light induced (Figure S4). This construct lacked endogenous 5′ and 3′ UTR sequences, strongly suggesting that the light regulation of HFR1 protein abundance occurred when the mRNA was constitutively present.

To explore the light regulation in more detail, we compared HFR1 protein accumulation between seedlings that were exposed to light for 2 hr and were then either transferred back to the dark or left in the light (compare Figures 1A and 1B). The accumulation of the two isoforms of HFR1 was parallel in the light, whereas upon transfer into darkness, the slow migrating form disappeared much faster than the other one (Figure 1). This experiment showed that HFR1 protein is downregulated with faster kinetics in the dark. This was particularly obvious for the slower migrating form, which was barely detectable after 1 hr in the dark but remained present during the entire time course in the light (compare Figures 1A and 1B).

### Regulation of HFR1 Stability by Phosphorylation and Light

Given that numerous transcription factors are phosphorylated and that two isoforms of HFR1 are present in the light, we tested if HFR1 was a phosphoprotein. HFR1-HA was immunoprecipitated, either from etiolated seedlings or from etiolated seedlings that were exposed to 2 hr of light, and treated with alkaline phosphatase (Figure 1C). After alkaline phosphatase treatment, HFR1-HA from light-treated plants migrated as a single band with the same mobility as the faster migrating band, indicating that the slower migrating form of HFR1 was phosphorylated (Figure 1C). HFR1 phosphorylation was not detectable in etiolated seedlings in the conditions used for this assay (see below). Taken together with the fast disappearance of the slowly migrating form in the dark (Figure 1B), our results suggested that phosphorylation of HFR1 rendered it particularly unstable in the dark.

To distinguish between light-regulated protein synthesis and protein stability, we analyzed HFR1-HA accumulation in the presence of cycloheximide in the dark and in the light. In the absence of protein synthesis, HFR1 disappeared very rapidly in the light and in the dark, indicating that it was unstable in both conditions (Figure 2). However, the protein was significantly more

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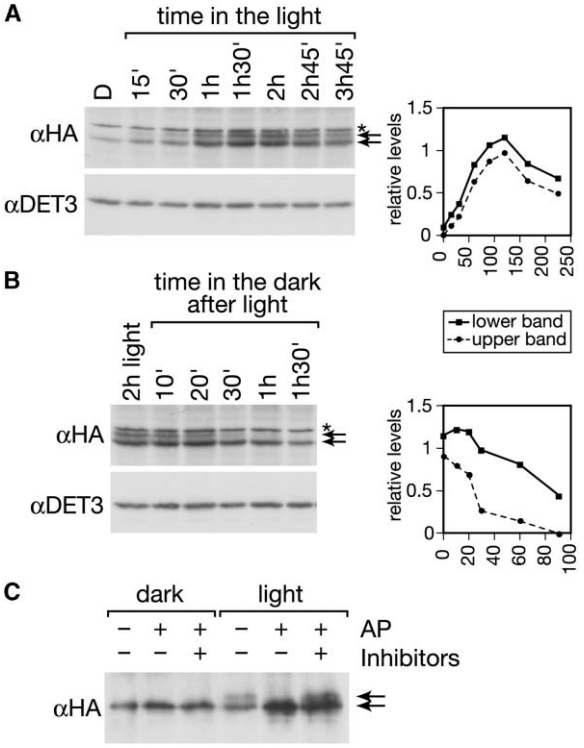


Figure 1. HFR1 Protein Accumulates in Response to Light and Is Phosphorylated

(A) Four-day-old etiolated transgenic seedlings expressing the HFR1-HA midi-gene were exposed to constant light (30 μE, blue), and total proteins were extracted at the indicated times. HFR1-HA accumulation was analyzed by Western blotting using anti-HA antibodies, and DET3 accumulation was used as loading control. The asterisk indicates the cross-reactive band also appearing in *hfr1* and wild-type extracts (Figure S2). The arrows indicate the two forms of HFR1. The right panels show the quantification of the upper and lower band relative to DET3.

(B) Four-day-old etiolated transgenic seedlings expressing the HFR1-HA midi-gene were exposed to constant light (30 μE, blue) for 2 hr and transferred back into darkness, and seedlings were harvested at the indicated times. HFR1 protein accumulation was analyzed as described in panel A. Note that the time 0 in panel B corresponds to the 2 hr point in panel A.

(C) Four-day-old etiolated transgenic seedlings expressing the HFR1-HA midi-gene were exposed for 2 hr to light (30 μE, blue) or kept in darkness. HFR1-HA was immunoprecipitated from a total extract using anti-HA antibodies and anti-mouse IgG antibodies coupled to magnetic beads (Dynabeads M-280 Sheep anti-mouse IgG, Dynal biotech). After washing, the beads were treated as described in the Supplemental Data. AP stands for alkaline phosphatase, and inhibitors are phosphatase inhibitors (Experimental Procedures).

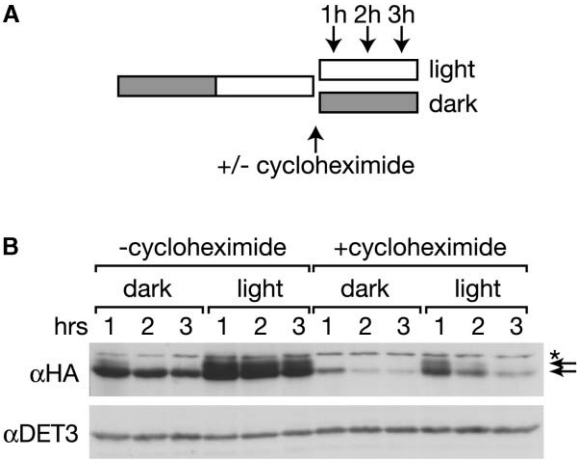


Figure 2. HFR1 Protein Is Highly Unstable

(A) Scheme of the experimental protocol.

(B) Four-day-old etiolated transgenic seedlings expressing the HFR1-HA midi-gene were exposed to light (30 μE, blue) for 2 hr before transferring them back into the dark or keeping them in the light in the presence or absence of 100 μM of cycloheximide. Control samples were treated with 1.4% ethanol. Total proteins were extracted at the indicated times and analyzed by Western blotting using anti-HA antibodies. DET3 accumulation was used as loading control.

stable in the light, indicating that the stability of HFR1 is light regulated (Figure 2B).

### Degradation of HFR1 Requires the 26S Proteasome

The proteasome system degrades unfolded proteins or removes proteins as a mechanism of down-regulation of certain signaling cascades [16]. To test if HFR1 is degraded through the 26S proteasome, 35S:HFR-HA seedlings were treated with proteasome inhibitors in the light and in the dark (Figure 3A). Immunoblot analyses indicated that the presence of proteasome inhibitors prevented HFR1 degradation in vivo, particularly in the dark (Figure 3B). The highly labile phosphorylated form in the dark was strongly stabilized in the presence of proteasome inhibitors (Figure 3B). This result suggested that in the dark, the phosphorylated form of HFR1 was most likely degraded through the 26S proteasome.

### The N-Terminal Domain of HFR1 Is a Determinant of Protein Stability

Several bHLH class proteins including HFR1, PIF1, PIF3, PIF4, PIL1, and PIL6 have been implicated in light signaling [7, 10–12, 14, 17]. Sequence alignments indicate that they all belong to the same evolutionary clade and that in addition to the bHLH domain, these proteins share a short stretch of homology in the amino-terminal region [13, 14, 18]. These observations prompted us to analyze the function of this domain. For this purpose we generated HFR1-carrying transgenic lines deleted for the amino-terminal domain (35S:ΔN-HFR1-HA). When the light regulation of ΔN-HFR1-HA protein was analyzed by immunoblotting, we noted that deletion of this amino-terminal domain led to stabilization of the protein, particularly in the dark (Figure 3C). In contrast to full-length HFR1, we could only detect a single isoform of ΔN-HFR1-HA (Figures 4C and 4D). This suggested that deleting this domain removed phosphorylation sites and/or that the change in HFR1 size rendered it more difficult to separate different isoforms. In order to test if ΔN-HFR1-HA was still unstable, seedlings expressing this construct were treated with cycloheximide. In the presence of protein synthesis inhibitor, the instability of ΔN-HFR1-HA could be detected (Figure 3D). Simultaneous treatment with cycloheximide and proteasome inhibitors stabilized the protein (Figure 3D). These experiments indicated that ΔN-HFR1-HA was more stable than

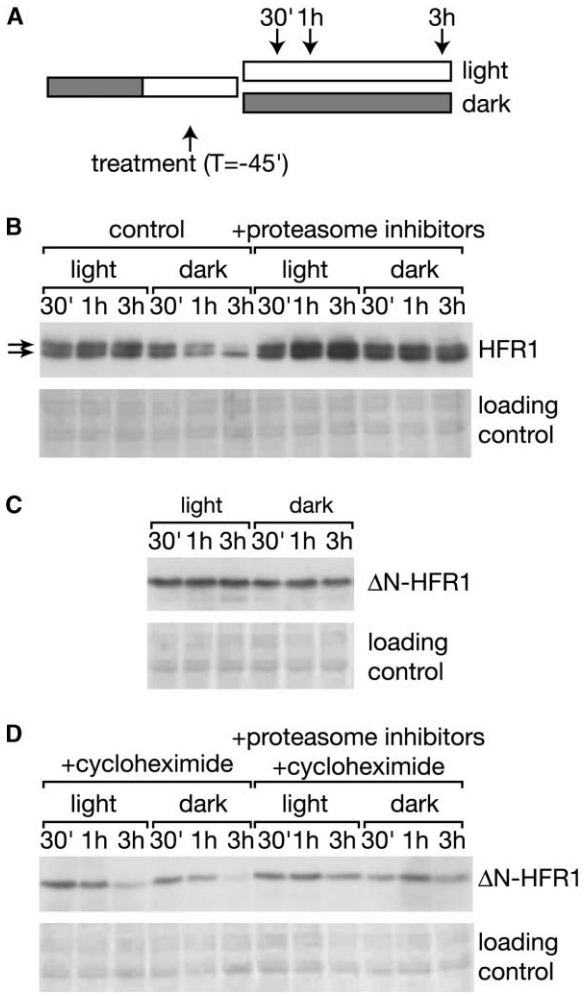


Figure 3. HFR1 Is Degraded through the 26S Proteasome and the N-Terminal Domain Destabilizes the Protein

(A) Scheme of the experimental protocol.

(B) Four-day-old etiolated transgenic seedlings expressing HFR1-HA under the control of the 35S promoter were exposed to light (30 μE, blue) for 90 min before adding the proteasome inhibitors ALLN, MG115, MG132, and PS1 (50 μM each). Control seedlings were treated with 3.5% DMSO. Forty-five minutes later, they were either kept in the light or transferred back into the dark. Total proteins were extracted at the indicated times and analyzed by Western blotting with anti-HA antibody. Membranes stained with Ponceau S are shown as a loading control.

(C) Four-day-old etiolated transgenic seedlings expressing ΔN-HFR1-HA under the control of the 35S promoter were exposed to light (30 μE, blue) for 2 hr before being transferred back into the dark or kept in the light. Total proteins were extracted at the indicated times and analyzed by Western blotting with anti-HA antibody.

(D) Transgenic seedlings expressing ΔN-HFR1-HA under the control of the 35S promoter were exposed for 90 min to the light before adding 100 μM of cycloheximide with or without the proteasome inhibitors ALLN, MG115, MG132, and PS1 (50 μM each). Forty-five minutes later, they were either kept in the light or transferred back into darkness. Total proteins were extracted at the indicated times and analyzed by western blotting with anti-HA antibody. Membranes stained with Ponceau S are shown as a loading control.

HFR1-HA but was still degraded by the proteasome (Figure 3).

The complex light regulation of HFR1 stability suggests that this mechanism may be required for a normal de-etiolation response. In order to test this, we characterized transgenic lines overexpressing either full length HFR1 (35S:HFR1-HA) or the more stable form of HFR1 lacking the amino-terminal domain (35S:ΔN-HFR1-HA) (Figure S5). Because HFR1 is involved in phyA and cry1 signaling, we studied the de-etiolation response in both far-red and blue light [9]. Lines strongly expressing each construct had shorter hypocotyls than the wild-type in both light conditions (Figure S5 and data not shown). These data suggested that an increased level of HFR1 leads to an exaggerated light response and that the tight control of HFR1 protein abundance is important for a normal de-etiolation response. In contrast to the results reported about overexpression of HFR1 lacking the first 105 amino acids, overexpression of our construct (lacking the first 49 amino acids) did not lead to a de-etiolation phenotype in the dark (Figure S5) [19]. Given that our construct still displayed some light-regulated accumulation (data not shown), this difference might be due to a greater stability of HFR1Δ105 compared to HFR1Δ49. Alternatively, different HFR1 amino-terminal deletions may have distinct functional consequences.

### COP1 controls HFR1 degradation

Several results have suggested that the E3 ligase COP1 might be involved in HFR1 degradation. First, *hfr1* partially suppresses the *cop1* phenotype [20]. Second, the phenotype of etiolated transgenic lines overexpressing HFR1 lacking the first 105 amino acids show a *cop1* like phenotype [19]. Finally, other positive components of phytochrome and cryptochrome signaling were shown to be substrates of COP1 E3 ligase activity in vitro [4–6]. To analyze if COP1 controls HFR1 degradation, we crossed the *cop1-4* mutant with HFR1-HA transgenic lines and compared isogenic siblings in the presence and absence of COP1. In the *cop1-4* mutant background, the light regulation of HFR1 accumulation was greatly impaired (Figure 4A). In etiolated seedlings, HFR1 accumulated to higher levels in the *cop1-4* background, suggesting that COP1 limited HFR1 accumulation in the dark (Figure 4A). In the wild-type, only the unphosphorylated form could be detected, whereas in *cop1* mutants, the two HFR1 isoforms accumulated to high levels (Figure 4A). Upon transfer into the light for 2 hr, the levels of HFR1 remained stable in the *cop1* mutant background, suggesting that in the wild-type, light protected HFR1 from COP1-dependent degradation (Figure 4A). Upon return into darkness, the two HFR1 isoforms remained relatively stable in the *cop1* mutant, in contrast to the wild-type, in which the phosphorylated form disappeared preferentially (Figure 4A). These results suggested that phosphorylation of HFR1 targets it for degradation in a COP1-dependent mechanism. Moreover, they suggested that phosphorylation is not light dependent per se but that phosphorylated HFR1 cannot be detected in etiolated wild-type seedlings because it gets degraded too rapidly. To test this hypothesis, we analyzed HFR1 protein in etiolated seedlings treated with proteasome inhibitor drugs. As expected, the slower migrating form of HFR1 could be detected in these conditions, consistent with the idea that phos-

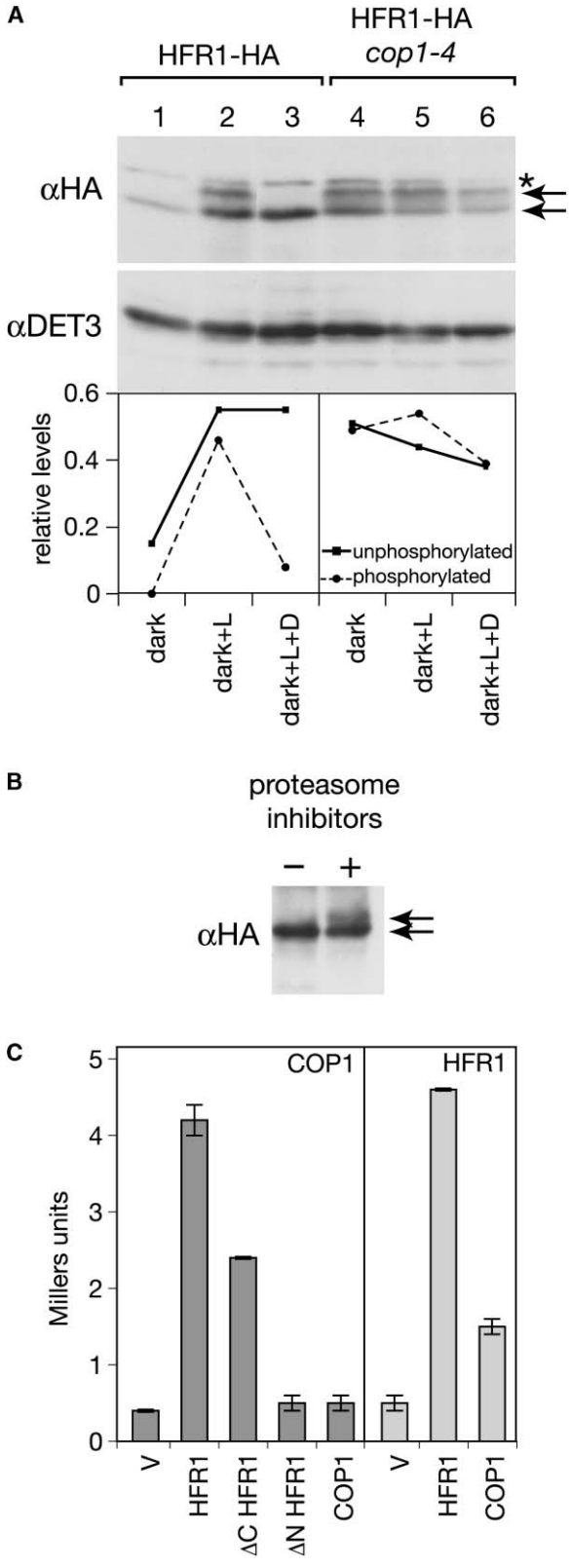


Figure 4. HFR1 Is More Stable in the Absence of COP1 and Interacts with COP1 in Yeast

(A) Analysis of HFR1-HA expression in the *cop1-4* background. Four-day-old transgenic seedlings expressing the HFR1-HA midi-gene and *cop1-4* transgenic seedlings expressing the HFR1-HA midi-gene were compared in etiolated seedlings (1 and 4), after exposure to 2 hr of light (100 μE, white) (2 and 5), and after exposure to 2 hr of light (100 μE, white) followed by a return to darkness for 3 hr (3 and 6). Total proteins were extracted and the Western blot probed either with the anti-HA antibody or with an antibody directed against DET3 as a loading control. The lower panel shows the quantification of HFR1-HA levels relative to DET3.

(B) Four-day-old etiolated transgenic seedlings expressing the HFR1-HA midi-gene were treated with proteasome inhibitors (or mock treated) in the dark during 2 hr before immunoprecipitation with anti-HA antibodies in the presence of proteasome inhibitors (as described in the legend for Figure 3). The immunoprecipitated proteins were separated by SDS-PAGE and Western blotted. Immunoblots were probed with the anti-HA antibody.

(C) HFR1 and COP1 interact in yeast. Yeast cells were cotransformed with the indicated prey and bait plasmids, and the interaction of the fusion proteins was monitored by quantitative liquid β-galactosidase activity in a yeast two-hybrid system [30]. The baits were fusions of the LexA DNA binding domain with either COP1 (left) or HFR1 (right). Prey proteins were HFR1 full length (HFR1), N-terminal 132 amino acids of HFR1 (ΔC HFR1), HFR1 lacking 132 N-terminal amino acids (ΔN HFR1) and COP1. The empty vector (V) was used as control. The results are presented as Miller's units in a quantitative liquid β-galactosidase assay. The average of two measurements ± SD is shown.

phorylation of HFR1 can occur in the dark and renders the protein more labile (Figure 4B).

The effect of COP1 on proteolytic degradation of HFR1 in vivo (Figure 4A) and the genetic interaction between *COP1* and *HFR1* [20] prompted us to test if the two proteins could interact. Our results indicate that HFR1 and COP1 did interact in the yeast two-hybrid assay (Figure 4C). This interaction occurred with a construct lacking the carboxy-terminus of HFR1 but not when the amino-terminus of HFR1 was absent (Figure 4C). Given that ΔN-HFR1-HA showed an increased stability in dark grown plants (Figure 3C), our results suggest that the amino-terminus of HFR1 might be a site of interaction with COP1. No perfect match to the published COP1 interaction motif was detected in HFR1, but related sequences are present in the amino-terminus of HFR1 (data not shown) [21]. Taken together, our results suggest that COP1 might be the E3 ligase required for ubiquitination of HFR1.

Similar genetic and molecular interactions have been described between the bZIP transcription factor HY5 and COP1. Moreover, COP1 has E3 ligase activity for HY5 in vitro and is required for efficient HY5 degradation in vivo [4, 22]. Like HFR1, HY5 is most effectively degraded in the dark and stabilized in the light. However, there are a number of differences between HFR1 and HY5 regulation. Although variations in HY5 levels take place within 5 hr, 24 hr of light or darkness is required to attain maximum or minimum levels of HY5, respectively [22]. The kinetics of HFR1 accumulation are much faster, since HFR1 reaches its maximum of abundance after 2 hr of light exposure and minimal levels of phosphorylated HFR1 after 90 min of darkness (Figure 1). Phosphorylation appears to have opposite effects on HFR1 and HY5 protein stability. HY5 phosphorylation stabilizes the protein [23], whereas phosphorylation of HFR1 renders the protein significantly less stable particularly in the dark (Figures 1, 3, and 4).

The effect of COP1 on HFR1 accumulation is particu-

larly obvious for phosphorylated HFR1 (Figure 4). Our data are compatible with a model in which phosphorylated HFR1 is turned over in a COP1-dependent manner and where light rapidly inhibits COP1 activity (Figure 4). This model is not consistent with the slow light-regulated nuclear depletion of COP1 that has been proposed to explain how light limits COP1 activity [24]. Our results therefore suggest that light controls COP1 activity at multiple levels. The cryptochromes have been suggested to rapidly inactivate COP1 upon light perception [25–27]. Given that HFR1 is a cryptochrome-signaling component, HFR1 might be a target of this short-term regulation. Furthermore, the activity and turnover of cry2 may similarly be coregulated by light [28]. Phosphorylation of the photoreceptor destabilizes the protein, and cry2 is also more stable in a *cop1* mutant background [28]. The SPA family of negative regulators of photomorphogenesis modulate the activity of COP1 [4, 5, 29]. It will be interesting to test if they are also involved in the regulation of HFR1 abundance.

We conclude that HFR1 protein abundance is tightly controlled by phosphorylation and light. Regulation of HFR1 abundance requires the E3 ligase COP1 in vivo, with the phosphorylated form of HFR1 being the preferred target for degradation. We identify a short amino-terminal domain present in multiple bHLH proteins involved in light signaling as a determinant for HFR1 stability. Furthermore, HFR1 and COP1 interact in yeast only when the N-terminal domain of HFR1 is present suggesting that this domain mediates the interaction between the two proteins.

## Experimental procedures

### Growth Conditions

Seeds of *Arabidopsis thaliana*, ecotype Columbia, were grown on 1/2 MS, 0.7% phytagar (Murashige and Skoog medium, GIBCO 23118-037) at 22°C in a Percival E-30LED with either the blue (λmax 469 nm), red (λmax 667 nm), or the far-red (λmax 739 nm) diodes in continuous light. Fluence rates were determined with an International light IL1400A photometer equipped with an SEL033 probe with appropriate light filters.

Generation of various constructs, generation of transgenic plants, plant protein extraction, immunoprecipitation, and yeast two-hybrid procedures are described in the Supplemental Experimental Procedures in the Supplemental Data.

### Supplemental Data

Supplemental Data including Supplemental Experimental Procedures and five additional figures are available at http://www.current-biology.com/cgi/content/full/14/24/2296/DC1/.

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