Reciprocal proteasome-mediated degradation of PIFs and HFR1 underlying photomorphogenic development in *Arabidopsis*

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**Summary Sentence:** The reciprocal degradation of PIFs and HFR1 highlights a novel mechanism by which HLH factors regulate the abundance of the bHLH factors to optimize photomorphogenesis in Arabidopsis.

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ABSTRACT

The phytochrome-mediated regulation of photomorphogenesis under red and far-red light conditions involves both positive and negatively acting factors. The positively acting factors (e.g., HY5/HFR1/LAF1 and others) are degraded in the dark to prevent photomorphogenesis. By contrast, the negatively acting factors (e.g., PIFs) are degraded in response to light to promote photomorphogenesis. Here we show that the negatively acting factor, PIF1 is also degraded in the dark by direct heterodimerization with the positively acting factor, HFR1. Conversely, PIF1 also promotes the degradation of HFR1 in darkness. PIF1 enhances the poly-ubiquitination of HFR1 by COP1 in vivo and in vitro. In addition, the reciprocal co-degradation of PIF1 and HFR1 is dependent on the 26S-proteasome pathway in vivo. Genetic evidence shows that the hfr1 mutant partially suppresses the constitutive photomorphogenic phenotypes of the cop1-6 pif1 and pifq both in the dark and far-red light conditions. Taken together, these data uncover a co-degradation mechanism between PIFs and HFR1 underlying photomorphogenic development in Arabidopsis thaliana.
INTRODUCTION

Plants undergo skotomorphogenic development in the dark, which is characterized by elongated hypocotyl and small appressed cotyledons. By contrast, they undergo photomorphogenic development under light, which is characterized by short hypocotyl, and expanded open cotyledons. Under red/far-red light conditions, photomorphogenesis is regulated by the phytochrome (phy) family photoreceptors (Bae and Choi, 2008; Wit, et al., 2016). Encoded by a small five-member family (phyA-phyE) in Arabidopsis, phys can form homo and heterodimers in vivo (Clack, et al., 2009). They are synthesized as inactive Pr form in the dark. Upon sensing red light using the billin chromophore, phys undergo a conformational change to a biologically active Pfr form that can be converted back to the Pr form by exposure to far-red light in a process called low fluence response (LFR). An exception among phys is phyA, for which a response can be triggered on exposure to very low amounts of any light (very low fluence response, VLFR) and for which continuous irradiation with high fluence rate far-red light will also trigger a response (high irradiance response, HIR) (Casal, et al., 2003). The Pfr form of all phys migrates into the nucleus with differential kinetics (Klose, et al., 2015), and regulates expression of a large number of genes to promote photomorphogenesis (Jiao, et al., 2007; Quail, 2007).

The phy-mediated light signaling pathways involve both positive and negatively acting factors (Huq and Quail, 2005). For example, HFR1/HY5/LAF1 and others are the major positive regulators (Lau and Deng, 2012; Xu, et al., 2016; Xu, et al., 2015), while PIFs act as major negative regulators of photomorphogenesis (Castillon, et al., 2007; Leivar and Monte, 2014; Leivar and Quail, 2011). PIFs consist of seven members (PIF1, PIF3-8), encoding basic helix-loop-helix (bHLH) family of transcription factors (Toledo-Ortiz, et al., 2003). They preferentially bind to the G-box (CACGTC) DNA sequence elements present in gene promoters and repress the light-inducible genes while activating the light-repressed genes in the dark (Kim, et al., 2016a; Leivar and Monte, 2014). A pifq mutant displayed constitutively photomorphogenic phenotypes suggesting that PIFs are promoting skotomorphogenesis (Leivar, et al., 2008; Shin, et al., 2009).
The 26S proteasome-mediated degradation of both positive and negatively acting factors plays a central role in the phy signaling pathways. In darkness, the positively acting factors (e.g., HY5/LAF1/HFR1 and others) are degraded (Lau and Deng, 2012). In this process, CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1), an E3 Ubiquitin ligase directly interacts with HY5/HFR1/LAF1 and induces degradation via the 26S proteasome pathway (Lau and Deng, 2012; Saijo, et al, 2003; Seo, et al, 2003; Xu, Paik, Zhu, et al, 2015). COP1 also associates with SUPPRESSOR OF PHYA-105 (SPA1-4) family members and CUL4, and the CUL4COP1-SPA complex degrades the positively acting factors to repress photomorphogenesis in the dark (Chen, et al, 2010; Hoecker, 2005; Saijo, Sullivan, Wang, et al, 2003; Zhu, et al, 2008). Strikingly, PIFs and the COP1/SPA complex function synergistically to degrade HY5 to repress photomorphogenesis in the dark (Xu, et al, 2014). Conversely, PIFs are phosphorylated, poly-ubiquitinated and subsequently degraded under light (Leivar and Quail, 2011; Xu, Paik, Zhu, et al, 2015). However, a recent study has shown that phyB can induce degradation of PIF1 non-cell autonomously (Kim, et al, 2016b). Among the candidate kinases, Oat phyA has been shown to directly phosphorylate PIFs and regulate the light-induced degradation of PIF3 under FR light (Shin, et al, 2016). CK2 and BIN2 have been shown to phosphorylate PIF1 and PIF4, respectively, in a light-independent manner (Bernardo-García, et al, 2014; Bu, et al, 2011b). In addition, both CUL3 and CUL4-based E3 ligases mediate the light-induced ubiquitination of PIF3 and PIF1, respectively, during dark to light transition (Ni, et al, 2014; Zhu, et al, 2015). HEMERA has also been shown to induce degradation of PIFs under de-etiolated conditions possibly in a transcription-coupled manner (Qiu, et al, 2015). However, PIFs are still degraded in these E3 ligase and kinase mutants, suggesting that additional factors are functioning in these processes.

Although, the positively acting factors are degraded in the dark, the dark-induced degradation of the negative factors has not been shown yet. Here we show that PIFs are degraded in the dark via 26S proteasome pathway. In this process, the positively acting factor, HFR1 promotes the degradation of PIF1 in the dark by direct heterodimerization. We further provide the biochemical and genetic evidence to support the hypothesis that PIF1 and HFR1 are undergoing reciprocal co-degradation via 26S proteasome pathway in the dark to optimize photomorphogenesis.
RESULTS

PIFs are degraded in the dark via 26S proteasome pathway

In general, PIFs are stable in the dark and light exposure induces their rapid degradation via 26S proteasome pathway (Castillon, Shen and Huq, 2007; Leivar and Quail, 2011; Xu, Paik, Zhu, et al, 2015). To test if PIFs are also degraded in the dark, we examined TAP-PIF1, PIF3-myc, PIF4-myc, PIF5-myc and native PIF1 and PIF5 levels in the dark treated without or with the proteasome inhibitor for 3 hours. The results show that the proteasome inhibitor treatment stabilized all four PIFs (Fig. 1A, B; Supplementary Fig. 1A, B, C). We also examined PIF1 protein level from wild type dark-grown seedlings with the protein synthesis inhibitor. Strikingly, the data show that PIF1 is rapidly degraded in the dark in the absence of new protein synthesis (Supplementary Fig. 1A). Although cellular proteins have a finite half-life, inhibition of PIF degradation by the proteasome inhibitor suggests that PIFs are degraded either directly or indirectly by the 26S proteasome pathway in the dark. These data sharply contrast the prevailing view that PIFs are only degraded in response to light.

Previously, both PIF1 and PIF3 have been shown to be unstable in the cop1-4 in the dark (Bauer, et al, 2004; Zhu, Bu, Xu, et al, 2015). We have examined PIF5 level in the cop1-4 seedlings grown in the dark. Similar to PIF1 and PIF3, PIF5 level is also lower in the cop1-4 compared to wild type seedlings (Supplementary Fig. 1C). To examine if the degradation of PIF1 and PIF5 in the cop1-4 depends on the 26S proteasome, we treated cop1-4 dark-grown seedlings with the proteasome inhibitor and measured PIF1 and PIF5 levels by immunoblots. Results show that both PIF1 and PIF5 are strongly stabilized in the cop1-4 by the proteasome inhibitor (Supplementary Fig. 1B, C), suggesting that PIF1 and PIF5 might be actively degraded in the cop1-4 background.

HFR1 promotes the degradation of PIF1 and PIF5

A recent study showed that overexpression of HECATE2 (HEC2), a HLH transcription factor stabilizes PIF1 in the dark and reduces the light-induced degradation of PIF1 (Zhu, et al, 2016). HFR1, another HLH factor was originally identified as an important positive regulator of phyA–mediated far-red light signaling and shade avoidance pathways (Fairchild, et al, 2000; Fankhauser and Chory, 2000; Hersch, et al, 2014; Lorrain, et al, 2009). To examine if HFR1 regulates PIF1 level, we performed immunoblots to examine PIF1 level in the hfr1 background under dark conditions. Results show that PIF1 is stabilized
in the hfr1 background under darkness (Fig. 2A, B). PIF5 is also slightly stabilized in the hfr1 single mutant under dark similar to PIF1 (Supplementary Fig. 1D). The HFR1-mediated PIF1 degradation is posttranslational as PIF1 mRNA level is slightly lower in the hfr1 mutant compared to wild type seedlings (Supplementary Fig. 1E). These data suggest that HFR1 promotes the degradation of PIF1 and PIF5 under dark.

Previous studies showed that COP1 promotes the degradation of HFR1 via 26S proteasome pathway in the dark (Jang, et al., 2005; Yang, et al., 2005b). Because PIF1 can interact with HFR1 and COP1 directly (Bu, et al., 2011a; Shi, et al., 2013; Xu, Paik, Zhu, et al., 2014; Yang, Lin, Sullivan, et al., 2005b), it is possible that COP1, PIF1 and HFR1 form a trimolecular complex that promotes the degradation of PIF1 in the dark. To test this hypothesis, we examined PIF1 level in the cop1-4, and cop1-4 hfr1 backgrounds under dark conditions (Fig. 2B). As expected, PIF1 level is lower in the cop1-4 in darkness compared to wild type. Strikingly, in the dark, PIF1 is strongly stabilized in the cop1-4 hfr1 compared to cop1-4 and wild type backgrounds (Fig. 2B). In addition, PIF5 level is also higher in the cop1-4 hfr1 compared to cop1-4 and wild type seedlings grown in darkness (Supplementary Fig. 1D). These data strongly suggest that HFR1 promotes PIF1 and PIF5 degradation in the wild type as well as cop1-4 backgrounds in the dark. Because cop1-4 expresses a truncated COP1 protein that was shown to retain residual function (McNellis, et al., 1994), the strongly higher abundance of PIF1 and PIF5 in the cop1-4 hfr1 seedlings compared to cop1-4 and hfr1 suggest that COP1 is required for the HFR1-mediated PIF1 and PIF5 turnover in the dark. Consistent with this conclusion, PIF1 level is slightly higher in the cop1-5, a null allele of cop1 mutant (Supplementary Fig. 1F).

**PIF1-HFR1 heterodimerization is necessary for the HFR1-mediated PIF1 degradation**

HFR1 heterodimerizes with PIFs to inhibit their activity. The substitution mutations of Val172Leu173 to Asp172Glu173 in the HLH domain of HFR1 have been shown to eliminate the dimerization between HFR1 and PIF1/PIF4/PIF5 (Supplementary Fig. 2A) (Hornitschek, et al., 2009; Shi, Zhong, Mo, et al., 2013). To test if the heterodimerization is necessary for the HFR1-mediated degradation of PIF1, we created a mutant version of HFR1 (HFR1*) that interferes with the dimerization between HFR1 and PIF1 as shown previously. Using yeast two-hybrid assays, we confirmed that the mutant HFR1* indeed lacks interaction with PIF1 (Supplementary Fig. 2B). We made transgenic plants expressing GFP-HFR1* in the hfr1 background and selected homozygous lines expressing similar amounts of the mutant and wild type GFP-HFR1. We also performed immunoblots to examine if HFR1* is degraded
in the dark similar to the wild type HFR1 as previously reported (Jang, Yang, Seo, et al., 2005; Yang, et al., 2005a). Interestingly, the data show that GFP-HFR1* level is similar under both dark and dark to light transition (Supplementary Fig. 3A-C). In contrast, GFP-HFR1 is degraded in the dark, but stabilized under light as previously reported. These data suggest that dimerization is necessary for degradation of HFR1 in the dark.

We then crossed both the wild type GFP-HFR1 and the mutant GFP-HFR1* into the cop1-4 hfr1 background. Phenotypic analyses showed that GFP-HFR1 suppressed the hypocotyl lengths of the hfr1 and cop1-4 hfr1 under far-red light, but the GFP-HFR1* failed to reduce the hypocotyl lengths of the hfr1 and cop1-4 hfr1 under these conditions (Supplementary Fig. 3C-E; 4A-D), confirming that the mutant HFR1* is non-functional in vivo as previously reported (Hornitschek, Lorrain, Zoete, et al., 2009; Shi, Zhong, Mo, et al., 2013). We then examined PIF1 level in the cop1-4 hfr1/GFP-HFR1 and cop1-4 hfr1/GFP-HFR1* by immunoblot. Strikingly, the GFP-HFR1 in the cop1-4 hfr1 background reduced PIF1 level close to the wild type (Fig. 2C, D). In contrast, GFP-HFR1* failed to reduce PIF1 level, suggesting that HFR1 promotes PIF1 degradation in the dark in a heterodimerization-dependent manner.

Our data along with others show that PIF1, PIF3 and PIF5 levels are lower in the cop1-4 compared to wild type at the seedlings stage (Supplementary Fig. 1) (Bauer, Viczian, Kircher, et al., 2004; Zhu, Bu, Xu, et al., 2015). However, in imbibed seeds, PIF1 level is much higher in the cop1-4 and spaq compared to wild type (Zhu, Bu, Xu, et al., 2015). To test if the expression level of HFR1 contributes to this difference, we measured HFR1 and PIF1 mRNA levels in both the wild type and cop1-4 imbibed seed and four-day-old dark-grown seedlings using RT-qPCR. Results show that HFR1 is strongly expressed in dark-grown seedlings with very weak expression in imbibed seeds while PIF1 is strongly expressed in imbibed seeds (Supplementary Fig. 5A, B). HFR1 expression is slightly lower in the cop1-4 seedlings compared to wild type seedlings, but still much higher than HFR1 level in seed stage (Supplementary Fig. 5B). These data suggest that the lower level of PIFs in the cop1-4 dark-grown seedlings might be due to the increased abundance of HFR1 that promotes the degradation of PIFs in the cop1-4 background. Taken together, these data demonstrate that HFR1 regulates PIF level in the dark in both wild type and cop1-4 backgrounds.
HFR1 promotes PIF1 degradation via 26S proteasome

To examine if HFR1 mediated degradation of PIF1 is proteasome-dependent, we created transgenic plants expressing TAP-PIF1 in the cop1-4 and cop1-4 hfr1 mutants and performed immunoblot in the presence and absence of the proteasome inhibitor. Results show that the TAP-PIF1 degradation is blocked in the presence of the proteasome inhibitor both in the cop1-4 and cop1-4 hfr1 mutant backgrounds under darkness similar to wild type background (Figs. 1, 3A-B). In addition, TAP-PIF1 is higher in the cop1-4 hfr1 background compared to that in the cop1-4 background (Fig. 3A-B), which is consistent with the native PIF1 level (Fig. 2B, C). These data suggest that HFR1 promotes the degradation of PIF1 via 26S proteasome pathway.

Recently, it was shown that some proteins are degraded via 26S proteasome pathway independent of polyubiquitination due to the presence of an unstructured region or through interaction with another protein containing an unstructured region (Fishbain, et al, 2015). To distinguish if the HFR1-mediated degradation of PIF1 is polyubiquitin-dependent or independent, we immunoprecipitated TAP-PIF1 from the cop1-4 and cop1-4 hfr1 mutants pretreated with the proteasome inhibitor and then detected with anti-Myc and anti-Ub antibodies. Results show that the immunoprecipitated TAP-PIF1 level is significantly higher in the cop1-4 hfr1 background than that in the cop1-4 background as observed (Fig. 3 C, left panel; 3D). But the ubiquitination level of the immunoprecipitated TAP-PIF1 is significantly lower in the cop1-4 hfr1 background than that in the cop1-4 background (Fig. 3C, right panel; 3D). The immunoprecipitated TAP-PIF1 is more abundant but contains less polyubiquitination in the cop1-4 hfr1 than in the cop1-4 background, which supports the hypothesis that HFR1 promotes the degradation of PIF1 in the dark via 26S proteasome pathway by increasing the amount of polyubiquitination of PIF1.

PIFs promote the degradation of HFR1 posttranslationally

COP1-SPA complex interacts with HFR1 and induce its degradation via 26S proteasome pathway in the dark (Jang, Yang, Seo, et al, 2005; Yang, Lin, Sullivan, et al, 2005b). COP1-SPA complex and PIFs also synergistically suppress plant photomorphogenesis in the dark by regulating the abundance of HY5 posttranslationally (Xu, Paik, Zhu, et al, 2014). To determine if the synergistic promotion of photomorphogenesis observed in the cop1-6 pif1 mutant is also partially due to an increased abundance of HFR1,
we generated the GFP-HFR1 transgenic plants in the *pif1*, *cop1-6* and *cop1-6 pif1* by crossing GFP-HFR1 into these backgrounds, respectively. Immunoblots showed that in both darkness and far-red light conditions, the GFP-HFR1 protein is synergistically stabilized in the *cop1-6 pif1* compared with that of the GFP-HFR1 in *pif1* and *cop1-6* single mutant backgrounds, respectively (Fig. 4A, B). This regulation is at the posttranslational level as the amount of the *GFP-HFR1* mRNA is similar in these backgrounds (Supplementary Fig. 6A). In addition, since *pifq* displays constitutive photomorphogenic phenotypes similar to *cop1*, we further created GFP-HFR1 transgenic plants in the *pifq* background. Strikingly, the GFP-HFR1 protein level, but not the *GFP-HFR1* mRNA level, is increased in the *pifq* compared to the wild type background (Fig. 4C, D; Supplementary Fig. 6A). A recent study also showed that the *HFR1* mRNA level is reduced in the *pifq* compared with the wild type (Supplementary Fig. 6B) (Zhang, *et al*., 2013), suggesting that PIFs also transcriptionally activate the expression of *HFR1*. Taken together, these data suggest that HFR1 abundance is also regulated by PIFs and COP1 in a posttranslational manner.

**PIF1 promotes HFR1 degradation via 26S proteasome**

Since HFR1 promotes PIF1 degradation by polyubiquitination via 26S proteasome pathway (Fig. 3), we hypothesized that PIFs promote HFR1 degradation in a similar manner. To examine if PIF-mediated degradation of HFR1 is proteasome-dependent, we first performed immunoblot for GFP-HFR1 in the presence and absence of the proteasome inhibitor. Results show that the GFP-HFR1 degradation is blocked in the presence of the proteasome inhibitor in the *GFP-HFR1* background under dark (Fig. 5A-B). The proteasome inhibitor also blocked GFP-HFR1 degradation in the *pifq* background but to a lesser degree compared to the *GFP-HFR1* background (Fig. 5A-B). Then, we immunoprecipitated GFP-HFR1 fusion protein from *GFP-HFR1* and *pifq/GFP-HFR1* transgenic seedlings pretreated with proteasome inhibitor and then detected with anti-Ub and anti-GFP antibodies. Results show that the immunoprecipitated GFP-HFR1 level is significantly higher in the *pifq/GFP-HFR1* than that in the *GFP-HFR1* as observed above (Fig. 5C, left panel, 5D). However, the polyubiquitination level of the immunoprecipitated GFP-HFR1 is significantly reduced in the *pifq* background than that in the *GFP-HFR1* background (Fig. 5C, right panel, 5D). These data support the hypothesis that PIFs promote the degradation of HFR1 in the dark via polyubiquitination followed by 26S proteasome pathway *in vivo.*
PIF1 enhances COP1-mediated ubiquitination of HFR1

COP1 directly ubiquitinated HFR1 in vitro (Jang, Yang, Seo, et al., 2005; Yang, Lin, Sullivan, et al., 2005b). The polyubiquitination level is also reduced in the pifq background in vivo as shown above (Fig. 5C, D), suggesting that PIFs might enhance the ubiquitination activity of COP1 toward HFR1. To test this hypothesis, we performed an in vitro ubiquitination assay as described previously (Jang, Yang, Seo, et al., 2005; Xu, Paik, Zhu, et al., 2014; Yang, Lin, Sullivan, et al., 2005b) using MBP-COP1, GST-HFR1 and different amounts of MBP-PIF1 or MBP as a control. Results show that COP1 functions as an E3 ligase to polyubiquitinate HFR1 as previously reported (Fig. 5E, lane 3) (Jang, Yang, Seo, et al., 2005). In addition, PIF1 promotes the polyubiquitination of HFR1 by COP1 in a concentration-dependent manner (Fig. 5E, lanes 4 and 5). In contrast, the addition of the MBP control protein did not affect the COP1-mediated ubiquitination of HFR1 (Fig. 5E, lane 6). Taken together, these results demonstrate that PIF1 promotes the COP1-mediated polyubiquitination of HFR1.

hfr1 partially suppresses the cop1-6 pif1 and pifq phenotypes

PIFs and HFR1 have a long history of antagonistic functions in regulating seedling deetiolation, seed germination and shade avoidance phenotypes (Castillon, et al., 2009; Duek and Fankhauser, 2003; Fairchild, Schumaker and Quail, 2000; Fankhauser and Chory, 2000; Hersch, Lorrain, de Wit, et al., 2014; Lorrain, Trevisan, Pradervand, et al., 2009; Oh, et al., 2004; Shi, Zhong, Mo, et al., 2013). To complement these published data, we examined if HFR1 can rescue the synergistic phenotype of the cop1-6 pif1, we generated a cop1-6 pif1 hfr1 triple mutant. Phenotypic analyses showed that the de-etiolated phenotypes are partially suppressed in the cop1-6 pif1 hfr1 triple mutant compared with those in the cop1-6 pif1 double mutant both in the dark and far-red light (Fig. 6A, B; Supplementary Fig. 7A-D). Since the partial suppression of the cop1-6 pif1 phenotype by hfr1 might be due to the hfr1’s suppression for cop1-6 only as shown previously (Kim, et al., 2002; Yang, Lin, Sullivan, et al., 2005b), we further created hfr1 pifq quintuple mutant. The constitutive photomorphogenic phenotypes of the pifq are also partially suppressed by hfr1 both under dark and far-red light (Fig. 6C). This could be due to suppression of other PIF activity as HFR1-mediated suppression of PIF7 has been shown previously (Hersch, Lorrain, de Wit, et al., 2014). Thus, these phenotypic data are consistent with the high abundance of GFP-HFR1 in cop1-6 pif1
and pifq backgrounds (Fig. 4; Fig. 5A, B). Because hfr1 suppresses pifq phenotype and conversely, pifq suppresses hfr1 phenotype under far-red light (Fig. 6C), the hyposensitive phenotype of hfr1 under far-red and blue light might be partly due to the higher amount of PIFs in the hfr1 background suppressing photomorphogenesis (Castillon, Shen and Huq, 2009; Duek and Fankhauser, 2003; Fairchild, Schumaker and Quail, 2000; Fankhauser and Chory, 2000). Taken together, these genetic and biochemical data suggest that HFR1 acts downstream of COP1 and PIFs in regulating photomorphogenesis.

Moreover, because HFR1 regulates seed germination under red light by controlling PIF1 activity (Shi, Zhong, Mo, et al, 2013), we also performed seed germination assays for hfr1 under an increasing fluence of far-red light conditions. hfr1 displayed reduced seed germination compared with wild type, suggesting that HFR1 also functions in phyA-dependent seed germination responses (Supplementary Fig. 8A). In addition, hfr1 pif1 double mutant displayed the same phenotype as pif1 single mutant, suggesting that pif1 is epistatic to hfr1 in phyA-dependent seed germination response. Consistent with the above phenotype, the expression of PIF1 target genes is increased in the hfr1 mutant background compared with the wild type both under dark and far-red light conditions (Supplementary Fig. 8B). These data further support the hypothesis that HFR1 promotes seed germination by regulating the abundance and the DNA binding activity of PIF1.

**DISCUSSION**

PIFs are known to be stable in the dark, and have been shown to undergo rapid degradation in response to red, far-red and blue light conditions (Leivar and Quail, 2011; Xu, Paik, Zhu, et al, 2015). In this process, phy interaction is necessary for the light-induced phosphorylation, polyubiquitination and subsequent degradation (Leivar and Quail, 2011). Both CUL3-LRB and CUL4-COP1-SPA complexes have been shown to function as E3 Ubiquitin ligases for the light-induced degradation of PIF3 and PIF1, respectively (Ni, Xu, Tepperman, et al, 2014; Xu, Paik, Zhu, et al, 2015; Zhu, Bu, Xu, et al, 2015; Zhu and Huq, 2014). In addition, DELLA proteins have been shown to promote degradation of PIFs independent of light (Li, et al, 2016). However, the degradation of PIFs in the dark has not been shown yet. Our data showing that PIFs are stabilized in the presence of proteasome inhibitor suggest that the abundance of PIFs is also regulated in the dark via 26S proteasome pathway. Thus, PIFs are posttranslationally regulated both in the dark and light.

Thus, HFR1 regulates PIF function not only by sequestration, but also by negatively regulating their abundance posttranslationally. The dual mechanisms ensure inhibition of PIF activity to optimize plant development in response to light. This is in contrast with another small family of HLH proteins named HECATE, which stabilizes PIF1 (Zhu, Xin, Bu, et al, 2016). Thus, bHLH-HLH interactions not only result in sequestration, but also posttranslational regulation of protein levels.

PIFs have been shown to display nontranscriptional roles in regulating HY5 posttranslationaly (Xu, Paik, Zhu, et al, 2014; Xu, Paik, Zhu, et al, 2015). We provide strong biochemical and genetic evidence that PIF1 and COP1 synergistically regulate HFR1 posttranslationally. Thus, PIF1 is acting as a cofactor for COP1 to regulate multiple COP1 substrates in vivo as predicted (Xu, Paik, Zhu, et al, 2015). By contrast, PIFs are not directly ubiquitinated by COP1 in vitro (Jang, et al, 2010; Xu, Paik, Zhu, et al, 2014; Zhu, Bu, Xu, et al, 2015). However, PIFs directly interact with COP1 and SPA1 in vitro and in vivo in the dark and light conditions (Jang, Henriques, Seo, et al, 2010; Xu, Paik, Zhu, et al, 2014; Zhu, Bu, Xu, et al, 2015). PIF1 is also poly-ubiquitinated by the CUL4-COP1-SPA complex in vivo (Zhu, Bu, Xu, et al, 2015). These data suggest a bifurcation of biochemical function where COP1 is sufficient to poly-ubiquitinate HFR1/HY5 and other positive factors in vitro, while COP1 might need to form CUL4-COP1-SPA complex to poly-ubiquitinate PIFs in vitro. Further biochemical assays using the CUL4-COP1-SPA complex are necessary to examine this hypothesis.
In summary, PIF1 and HFR1 are undergoing reciprocal degradation in the dark (Supplementary Fig. 9, left). Under red and far-red light, PIF1 is degraded by the CUL4-COP1-SPA complex, while HFR1 is stabilized by phy-mediated inhibition of COP1-SPA. The increased abundance of HFR1 sequesters residual PIF1 and other PIFs to promote seed germination and seedling de-etiolation under light (Supplementary Fig. 9, right). Recently, PIF3 and PhyB have been shown to undergo co-degradation in response to light via the CUL3\textsuperscript{LRB} complex (Ni, Xu, Tepperman, et al, 2014; Zhu and Huq, 2014). The co-degradation of PIF3 and PhyB appears to attenuate the incoming signals to protect plants by degrading the signal receptor as well as the primary signal acceptor in a mutually destructive manner (Ni, Xu, Tepperman, et al, 2014; Zhu and Huq, 2014). The co-degradation of PIF1 and HFR1 found in our study also demonstrates a similar mechanism in the dark, where photomorphogenesis would not be over repressed by an excessively high abundance of PIF repressors. This mechanism is important because elevated levels of PIF in the dark or during early light exposure distort seedling growth and gene expression during de-etiolation as has been shown previously (Khanna, et al, 2004; Krzymuski, et al, 2014).

MATERIALS AND METHODS

Plant materials, growth conditions and measurements

Seeds of wild type Col-0 and various mutant and tagged lines have been described (Castillon, Shen and Huq, 2009; Park, et al, 2004; Sakuraba, et al, 2014; Xu, Paik, Zhu, et al, 2014; Zhu, Bu, Xu, et al, 2015). The cop1-6 pif1 hfr1, cop1-6 hfr1, cop1-4 hfr1 and hfr1 pifq were generated by crossing hfr1 with cop1-6 pif1, cop1-6, cop1-4 and pifq. For generation of pif1 GFP-HFR1, pifq GFP-HFR1, cop1-6 GFP-HFR1, and cop1-6 pif1 GFP-HFR1, GFP-HFR1 was crossed into those mutant backgrounds. For generation of cop1-4 hfr1/GFP-HFR1 and cop1-4 hfr1/TAP-PIF1, cop-4 hfr1 was crossed into GFP-HFR1 and TAP-PIF1, respectively. The primers for genotyping were used as previously described (Castillon, Shen and Huq, 2009; Xu, Paik, Zhu, et al, 2014). To generate HFR1*GFP, HFR1* was first generated by site-directed mutagenesis with the primers listed in the Supplemental table (Shi, Zhong, Mo, et al, 2013). HFR1 ORF was cloned into pENTRY vector as previously described (Hornitschek, Lorrain, Zoete, et al, 2009). Then it was cloned into the GFP destination vector for transformation into hfr1 background as described (Bu, Castillon, Chen,
To generate cop1-4 hfr1 HFR1*GFP, cop1-4 hfr1 was crossed to the hfr1/HFR1*GFP.

Plants were grown in Metro-Mix 200 soil (Sun Gro Horticulture, Bellevue, WA) under 24-h light at 22 ± 0.5°C. Seeds were sterilized with bleach and then plated on the Murashige and Skoog medium supplemented 0.9% agar without sucrose as described (Shen, et al., 2005). After 3-4 days of cold treatment, seeds were exposed to white light for 3h at room temperature to trigger germination. For GFP-HFR1 immunoblot blot, seeds were either placed back to dark for 4 days or grown in the dark for 21h then transferred to continuous FRc (0.45 μmol/m^2/s) for 3 days. For PIF1 immunoblot, seeds were placed back to dark for 4 days to directly extract protein. For cycloheximide and proteasome inhibitor treatment, 4-day-old dark-grown seedlings were transferred to 5 mL media containing either 20 mM cycloheximide or 40 μM Bortezomib and incubated in the dark for the duration indicated in each figure as previously described (Shen, Moon and Huq, 2005; Zhu, Bu, Xu, et al., 2015). For gene expression and in vivo co-immunoprecipitation assays, seeds were placed back to dark for 4 days. For de-etiolation phenotypes, seedlings are grown either in the dark for 5 days or grown in the dark for 21h then transferred to FRc for 4 days before taking pictures. For measurement of hypocotyl lengths, cotyledon areas, and cotyledon angles, digital pictures of dark or FRc grown seedlings as mentioned above were taken and at least 30 seedlings were measured using ImageJ (http://rsb.info.nih.gov/ij/). The phenotypic assays were replicated as least three times. The phyA-dependent seed germination assays were performed as previously described (Zhu, Bu, Xu, et al., 2015).

**RNA isolation and quantitative RT-PCR**

The quantitative RT-PCR (RT-qPCR) for seedlings and seeds was performed as previously described (Xu, Paik, Zhu, et al., 2014; Zhu, Bu, Xu, et al., 2015; Zhu, Xin, Bu, et al., 2016). For seedlings, total RNA of 3 or 4-day-old dark-grown seedlings were extracted with Spectrum plant total RNA kit (Sigma-Aldrich Co., St. Louis, MO). For seeds, wild type and hfr1 were plated on MS plates with 100μM paclobutrazol within 1h and then treated with far-red light (34 μmol/m^2/s) for 5mins and kept in the dark for 2 days before RNA isolation. One μg of total RNA was used to reverse transcribe into cDNA using SuperScript III (Life Technologies Co., Carlsbad, CA) after DNase I treatment. RT-qPCR was performed using the Power SYBR Green Kit (Applied Biosystems Inc., Foster City, CA) in a 7900HT Fast Real-Time PCR machine. PP2A (At1g13320) was used as a control. The resulting cycle threshold
(Ct) values were used for calculation of the relative expression level for GFP genes relative to PP2A. The value of GFP-HFR1 was set as 1 to calculate the relative values of other genotypes. Primers of RT-qPCR are listed in the Supplemental table.

**Protein extraction and immunoblot analyses**

For GFP-HFR1 and native PIF1/5 immunoblots, seedlings were grown as described above. For TAP-PIF1, PIF3-Myc, PIF4-Myc and PIF5-myc immunoblots, plates were kept in darkness for 4 days, one batch of seedlings for each genotype was treated with proteasome inhibitor (40 μM Bortezomib) for 3h before protein extraction. Total protein was extracted in buffer (100 mM MOPS PH 7.6, 5% SDS, 10% glycerol, 40 mM EDTA pH 8, 1×protease inhibitor cocktail (Sigma-Aldrich Co., St. Louis, MO), 40 mM β-mercaptoethanol, 2 mM PMSF, 25 mM β-GP, 10 mM NaF and 2 mM Na-orthovanadate), followed by boiling in water for 3 mins. The samples were centrifuged at 16,000g for 10 min and then loaded the supernatant on 8% SDS-PAGE gel. After blotting onto polyvinylidene difluoride (PVDF) membranes, the same membrane was first blotted with anti-GFP, anti-PIF1 (Shen, et al, 2008), anti-PIF5 (Catalog# AS12 2112, Agrisera, Vännäs, Sweden) or anti-Myc (EMD Millipore, Billerica, MA) antibodies followed by anti-RPT5 or anti-actin antibody after stripping. For the quantification, we used ImageJ software to measure band intensities based on at least three independent blots.

**In vivo immunoprecipitation assays**

To detect the ubiquitination of TAP-PIF1 and GFP-HFR1 in pifq background in vivo, immunoprecipitation from 4-d-old dark-grown seedlings of each genotype were performed as previously described with minor modification (Shen, Ling, Castillon, et al, 2008). Briefly, total protein was extracted from ~0.4g 4-day-old dark grown seedlings pretreated with 40 μM Bortezomib for 3h before protein extraction. Total protein was extracted from seedling tissues (~0.4 g) with 1 mL urea extraction buffer (8M urea, 10mM Tris, pH 8.0, 100 mM NaH2PO4, 100mM NaCl, 0.05% Tween 20, 1×protease inhibitor cocktail [Sigma-Aldrich Co., St. Louis, MO], 2 mM PMSF, 40 μM Bortezomib, 25 mM β-glycerophosphate, 10 mM NaF, 2 mM Na-orthovanadate, and 100 nM calyculin A), and centrifuged in the dark at 16000g for 15 mins at 4°C. TAP-PIF1 or GFP-HFR1 was immunoprecipitated from the supernatant with Dynabeads Protein A bound to anti-Myc or anti-GFP antibodies, respectively. Then the pellets were washed and heated with SDS-buffer for 5 min at 65°C before loading to 6.5% SDS-PAGE gels. Same blot was first probed with anti-Ub antibody
followed by either anti-Myc (Mouse, EMD Millipore, Billerica, MA) or anti-GFP antibody after stripping for TAP-PIF1 or GFP-HFR1 blot, respectively.

**In vitro ubiquitination assays**

The *in vitro* ubiquitination assay was performed as previously described with minor modification (Jang, Yang, Seo, *et al.*, 2005; Xu, Paik, Zhu, *et al.*, 2014; Yang, Lin, Sullivan, *et al.*, 2005b). MBP-PIF1 was purified from *E. coli* as previously described (Xu, Paik, Zhu, *et al.*, 2014). HFR1 was digested from the HFR1-GAD (Castillon, Shen and Huq, 2009), and then cloned into pGEX4T-1 to obtain GST-HFR1. Both MBP-COP1 and GST-HFR1 proteins were purified from *E. coli* as previously described (Hardtke, *et al.*, 2000; Xu, Paik, Zhu, *et al.*, 2014). Flag-tagged ubiquitin (Flag-Ub), UBE1 (E1) and UbcH5b (E2) were used as previously described (Jang, Yang, Seo, *et al.*, 2005) (Boston Biochem, Cambridge, MA). For the *in vitro* ubiquitination reaction, 5 μg of Flag-Ubiquitin, ~25ng of E1, ~25ng of E2, ~500ng of MBP-COP1, ~200ng of GST-HFR1, and 50 or 100ng MBP-PIF1 were added in the reaction buffer containing 50 mM Tris, pH7.5, 2 mM ATP, 5 mM MgCl₂, and 2 mM DTT. MBP-COP1 was pretreated with 20 μM ZnCl₂ for 45min at 22°C before adding into the reaction system. Reactions were carried out at 30°C for 2h, and then the samples were heated at 95°C with SDS buffer. Reaction mixtures were then loaded onto 8% SDS-PAGE gel and blotted onto PVDF membrane. Ubiquitinated GST-HFR1 was first detected with α-Flag antibody (F1804; Sigma-Aldrich Co., St. Louis, MO) and same blot was then probed with anti-GST-HRP conjugate (GE Healthcare Bio-Sciences, Pittsburgh, PA).

**Yeast two hybrid analyses**

The full-length *HFR1, HFR1* and C-terminal DNA binding domain (bHLH) of PIF1 (C328) open reading frames (ORFs) were amplified by PCR using the primers listed in the Supplemental table 1. The entry clone containing HFR1* was used as the template to amplify mutant HFR1*. The PIF1-C328 clone has been described (Shen, Ling, Castillon, *et al.*, 2008). The full-length HFR1 and mutant HFR1* fragments were cloned into pGAD424 vector. These plasmids were transformed into yeast strain Y187. A β-galactosidase activity assay for quantitation of the interaction was performed according to the manufacturer’s instructions (Matchmaker Two-Hybrid System; Clontech Laboratories).
Supplementary information

Supplementary information available online at http://dev.biologists.org/

Acknowledgments

We thank Drs. Xing Wang Deng for sharing the cop1 mutant, Haiyang Wang for GFP-HFR1 seeds and Giltsu Choi for PIF3-myc and PIF5-myc seeds. This work was supported by grants from the National Institute of Health (NIH) (1R01 GM-114297) and National Science Foundation (MCB-1543813) to E.H.

Author Contributions

References:


Fig. 1: PIF1, PIF3, PIF4 and PIF5 are degraded in the dark via 26S proteasome pathway.

(A) Immunoblots showing the PIFs level in 4-day-old pPIF1:TAP-PIF1, 35S:PIF3-Myc, 35S:PIF4-Myc and 35S:PIF5-Myc dark-grown seedlings. One batch of seedlings was pretreated with 40 μM Bortezomib, Bortz for 3h before protein extraction. pif1, pif3, pif4, and pif5 mutants were used as controls. The blot was probed with anti-Myc or anti-RPT5 antibodies. (B) Quantification of TAP-PIF1, PIF3-Myc, PIF4-Myc and PIF5-Myc protein levels using ImageJ. RPT5 was used as a control. The TAP-PIF1, PIF3-Myc, PIF4-Myc and PIF5-Myc protein levels without proteasome inhibitor treatment were set as 100 respectively. The error bars indicate standard deviation (n=3).
Fig. 2: HFR1 promotes PIF1 degradation in the dark.

(A) Immunoblot shows higher abundance of PIF1 in the hfrl and cop1-4hfrl backgrounds compared with wild-type seedlings. Four-day-old dark-grown seedlings were used for protein extraction. The blot was probed with anti-PIF1 and anti-RPT5 antibodies. (B) Quantification of PIF1 protein level using RPT5 as a control. The letters “A” to “D” indicate statistically significant differences between means of protein levels (p<0.05) based on two-way ANOVA analyses. The error bars indicate standard deviation (n=7). (C) Immunoblots show the PIF1 (top panel) and GFP-HFR1 (middle panel) and loading control RPT5 (bottom panel) levels in wild type Col-0, cop1-4, cop1-4hfr1, cop1-4hfr1/GFP-HFR1 and cop1-4hfr1/GFP-HFR1*. Immunoblot was performed as described (A). (D) Quantification of PIF1 protein level using RPT5 as a control. The letters “A” to “D” indicate statistically significant differences between means of protein levels (p<0.05) based on two-way ANOVA analyses. The error bars indicate standard deviation (n=3).
Fig. 3: HFR1-mediated PIF1 degradation is 26S proteasome dependent.

(A) Immunoblot shows the TAP-PIF1 level in cop1-4 and cop1-4hfr1 background. Total protein was extracted from 4-day-old dark-grown seedlings. One batch of seedlings was pretreated with 40 μM Bortezomib (Bortz) for 3h before protein extraction. The blot was probed with anti-Myc or anti-RPT5 antibodies. *, indicates a cross-reacting band or proteolytically cleaved product. (B) Quantification of TAP-PIF1 protein level using RPT5 as a control. The letters “A” to “C” indicate statistically significant differences among four samples and two treatment conditions (p<0.05) based on two-way ANOVA analyses. The error bars indicate standard deviation (n=3). (C) TAP-PIF1 level is higher but the ubiquitination level is lower in the cop1-4hfr1 compared with cop1-4 background in darkness. Total protein was extracted from 4-day-old dark-grown seedlings with 40 μM Bortezomib pretreatment for 3h before protein extraction. TAP-PIF1 was immunoprecipitated using anti-Myc antibody from protein extracts. The immunoprecipitated samples were then separated on 6.5% SDS-PAGE gels and probed with anti-Myc (left) or anti-Ub (right).
antibodies. The top and bottom panels are low and high exposures, respectively. Arrow indicates TAP-PIF1. (D) Quantification of TAP-PIF1 and TAP-PIF1-ubi protein levels shown in (C). The TAP-PIF1 and TAP-PIF1-ubi protein levels in cop1-4 background were set as 1 respectively. The error bars indicate standard deviation (n=3).
Fig. 4: PIFs promote the degradation of HFR1 posttranslationally in the dark and FR light.

(A) Immunoblot shows HFR1 protein level in the GFP-HFR1 transgenic plant and pif1, cop1-6 and cop1-6pif1, respectively, harboring the GFP-HFR1 transgene. Seedlings are grown either in the dark for 4 days or grown in the dark for 21h and then transferred to continuous FRc (0.45 μmol/m²/s) for 3 days. The blot was probed with anti-GFP or anti-RPT5 antibodies. (B) Bar graph shows GFP-HFR1 protein level in the mutants indicated. For quantitation, GFP-HFR1 band intensities were quantified from three independent blots using ImageJ, and then normalized against RPT5 levels. GFP-HFR1 dark level was set as 1 and the relative protein levels were calculated. Error bars indicate standard deviation. *, indicates significant difference (p<0.05) between double and single mutant background. (C) Immunoblot shows HFR1 protein level in the GFP-HFR1 and pifq/GFP-HFR1. An RPT5 blot shows a loading control. Seedlings were grown in the dark or FRc light as described above. (D) Bar-graph shows the quantified GFP-HFR1 levels in the GFP-HFR1 and pifq/GFP-HFR1. Error bars indicate standard deviation. *, indicates significant difference between GFP-HFR1 and pifq/GFP-HFR1 in both conditions, respectively (p<0.05).
Fig. 5: PIF1 promotes HFR1 degradation in a polyubiquitination-dependent manner.

(A) Immunoblot shows the GFP-HFR1 protein level in GFP-HFR1 and GFP-HFR1/pifq backgrounds. Total protein was extracted from 4-day-old seedlings grown in darkness. One batch of seedlings was pretreated with 40 μM Bortezomib (Bortz) for 3h before protein extraction. The blot was probed with anti-GFP or anti-Actin antibodies. (B) Quantification of GFP-HFR1 protein level using Actin as a control. The * indicate statistically significant differences compared with non-Bortezomib treatment for GFP-HFR1 and GFP-HFR1/pifq, respectively (p<0.05). The error bars indicate standard deviation (n=4). (C) The protein level of GFP-HFR1 is higher but the ubiquitination level of GFP-HFR1 is lower in the pifq compared with GFP-HFR1 background in darkness in vivo. Sample preparation is as described in (A). GFP-HFR1 was immunoprecipitated using anti-GFP antibody, and then separated on 8% SDS-PAGE gels and probed with anti-GFP (left) or anti-Ub (right) antibodies. The top and bottom panels are low and high exposures, respectively. The arrow indicates the GFP-HFR1 size. (D) Quantification of GFP-HFR1 and GFP-HFR1-ubi levels for (C) by ImageJ. The GFP-HFR1 and GFP-HFR1-ubi levels were set as 1, respectively. The
error bars indicate standard deviation (n=3). (E) PIF1 promotes the ubiquitination of HFR1 by COP1 in vitro. In Vitro Ubiquitination assay was performed using MBP-COP1 as E3 Ubiquitin ligase, GST-HFR1 as a substrate, Flag-Ubiquitin, UBE1 (E1), UbcH5b (E2), and increasing concentrations of MBP-PIF1. MBP was used as a control. Ubiquitinated GST-HFR1 detected by anti-Flag antibody (Top panel), and anti-GST antibody (Bottom panel). The arrow indicates non-ubiquitinated GST-HFR1.
Fig. 6: *hfr1* partially suppresses the phenotypes of *cop1-6pif1* and *pifq*.

(A and B) Photographs and bar graphs showing hypocotyl lengths of seedlings of wild type, *pif1*, *cop1-6*, *cop1-6pif1*, *cop1-6pif1hfr1*, *cop1-6hfr1*, *hfr1pif1* and *hfr1*. Seedlings were grown either in the dark for 5 days (A) or grown in the dark for 21 hours then transferred to continuous FRe (0.06 μmol/m²/s) for 4 days (B). White bar = 5 mm. Error bars indicate standard deviation. The letters “A” to “F” indicate statistically significant differences between means for hypocotyl lengths (p<0.05) based on two-way ANOVA analyses, (n>30, three biological replicates). (C) Photographs and bar graphs showing hypocotyl lengths of seedlings of wild type, *pifq*, *hfr1pifq*, and *hfr1*. Seedlings were grown either in the dark for 5 days (Top panel) or grown in the dark for 21h then transferred to FRe for 4 additional days. White bar = 5 mm. Error bars indicate standard deviation. *, indicates significant difference (p<0.05) compared with *pifq*. (n>30, three biological replicates).
Supplemental Figure 1: COP1 and HFR1 are involved in the 26S proteasome mediated degradation PIF1 and PIF5 in the dark.

(A) Immunoblot shows PIF1 level in 5-day-old wild type Col-0 dark-grown seedlings treated with 20 mM cycloheximide (CHX) or proteasome inhibitor (40 μM Bortezomib) for the indicated hours before protein extraction in the dark. CK is a control without any treatment in the dark. Total protein was separated on an 8% SDS-PAGE gel, blotted onto PVDF membrane and probed with anti-PIF1 or anti-RPT5 antibodies. (B) Immunoblot shows the PIF1 level in 4-day-old wild type Col-0 or cop1-4 dark-grown seedlings with and without proteasome inhibitor (40 μM Bortezomib) pretreatment for the indicated time before protein extraction in the dark. Total protein was separated on an 8% SDS-PAGE gel, blotted onto PVDF membrane and probed with anti-PIF1 or anti-RPT5 antibodies. (C) Immunoblot shows the PIF5 level in 4-day-old wild type Col-0 or cop1-4 dark-grown seedlings with and without proteasome inhibitor (40 μM Bortezomib) pretreatment for the 3 hours before protein extraction in the dark. Total protein was separated on an 8% SDS-PAGE gel, blotted onto PVDF membrane and probed with anti-PIF5 or anti-RPT5 antibodies. (D) Immunoblot shows the PIF5 level in 4-day-old wild type Col-0, cop1-4, hfr1, and cop1-4hfr1 dark-grown seedlings. Immunoblot was performed as described (C). (E) RT-qPCR data showing the relative expression of PIF1 in wild-type and hfr1-201 mutant. RNA was extracted from 4-day-old dark grown wild-type Col-0 and hfr1-201 seedlings and reverse transcribed into cDNA. (F) PIF1 is more abundant in cop1-5 compared to wild type. (Left) Immunoblot blot shows the PIF1 level in wild type Col-0 and cop1-5. Total protein was extracted from 4-day-old seedlings grown on the MS media in darkness. (Right) Quantification of PIF1 protein level using RPT5 as a control. * indicates statistically significant differences between means of protein levels (p<0.05). The error bars indicate standard deviation (n=3).
Supplemental Figure 2: **HFR1** does not interact with PIF1 in yeast 2-hybrid assays. A) The domain structure of HFR1. The N-terminal 131 domain of HFR1 is responsible for interaction with COP1 and triggered the 26 proteasome mediated degradation, the C-terminal 161 domain (CT161) is involved in forming heterodimer with PIF1/3/4/5 to block PIF’s transcriptional activity for binding to DNA. The ** indicate mutated version of the HFR1 protein (HFR1*) that substitutes two conserved residues Val172 Leu173 to Asp172Glu173 in the HLH domain, which can interfere with the dimerization. B) Quantitative yeast-two hybrid assay showing HFR1 directly interacts with the C-terminal bHLH domain of PIF1 (C328). PIF1 C328 was fused with GAL4 DNA binding domain (pGBT9). Full-length HFR1 and mutant HFR1* deficient in interaction with PIF1 were fused with GAL4 activation domain (pGAD424). Mutant HFR1* and empty vector (pGAD424) was used as negative control. AD: empty vector pGAD424. β-galactosidase units are Miller units. LacZ assays were performed in triplicate and error bars indicate standard deviation.
Supplemental Figure 3: HFR1* is stable in the dark and is non-functional in vivo.

(A) Immunoblot shows the GFP-HFR1 and GFP-HFR1* protein levels. Two batches of Arabidopsis seedlings expressing GFP-HFR1 or GFP-HFR1* were grown in the dark for 4 days and then one batch of seedlings was transferred to white light (WL) condition for 6 hours before total protein was extracted. Total protein was separated on 8% SDS-PAGE gel, blotted onto PVDF membrane and probed with anti-GFP or anti-Actin antibodies. (B) Quantification of GFP-HFR1 and GFP-HFR1* protein levels using Actin as a control. The letters “A” to “B” indicate statistically significant differences between means of relative protein levels of the indicated genotypes, (p<0.05). The error bars indicate standard deviation (n=3). C) Quantification of GFP-HFR1 and GFP-HFR1* mRNA levels using PP2A as a control in lines used in (A). Four-day-old dark-grown seedlings were used for RNA isolation. Error bars show standard deviation. ** p<0.01 (Student two-tailed t-test). D) Photographs of seedlings of various genotypes as indicated grown in the dark for 5 days or grown in the dark for 21 hours and then transferred to continuous FR light (0.45 μmol/m²/s) for 4 days. White bar=5mm. (E and F) Bar graphs showing the hypocotyl lengths for the seedlings grown in the dark (E) or far-red light (F). Error bars indicate standard deviation. The letters “A” to “E” indicate statistically significant differences between means for hypocotyl lengths (p<0.05), (n>30, three biological replicates).
Supplemental Figure 4: GFP-HFR1* does not rescue phenotype in the cop1-4 background.

(A and C) Photographs of seedlings of various genotypes as indicated grown in the dark for 5 days (A) or grown in the dark for 21 hours and then transferred to continuous FR light (0.45 μmol/m²/s) for 4 days (C). White bar=5mm. (B and D) Bar graphs showing the hypocotyl lengths for the seedlings shown in A and C. Error bars indicate standard deviation. The letters “A” to “E” indicate statistically significant differences between means for hypocotyl lengths (p<0.05), (n>30, three biological replicates).
Supplemental Figure 5: Expression of *PIF1* and *HFR1* in seeds and seedlings.

*PIF1* is expressed more in the seeds compared to seedlings (A), while *HFR1* is highly expressed at the seedling stage compared to seed stage (B). RT-qPCR data showing the relative expression of *PIF1* and *HFR1* in wild-type (Col-0) and *cop1-4* seedlings compared to seeds. RNA was extracted from 4-day-old dark grown wild-type Col-0, *cop1-4* seedlings and imbibed seeds. *PP2A* (At1g13320) was used as a control for normalization of the expression data. Inset in (B) shows *HFR1* expression in Col-0 and *cop1-4* seeds.
Supplemental Figure 6: GFP and native HFR1 mRNA levels in various backgrounds.

(A) Bar graph showing the GFP mRNA levels in the different genotypes as indicated. GFP mRNA level was determined using RT-qPCR assays using primers designed from the GFP region. Total RNA was isolated from 4-day-old dark-grown seedlings for RT-qPCR assays (n= 3 independent biological repeats). PP2A was used as an internal control. GFP-HFR1 was set as 1 and the relative gene expression levels were calculated. Error bars indicate standard deviation. (B) Bar graph shows the native HFR1 mRNA level in the wild type (Col-0) and pifq based on RNA-seq data as described {Zhang, 2013 #419}. Error bars indicate standard deviation. *, indicates significant difference (p<0.05).
Supplemental Figure 7: *hfr1* partially suppresses the synergistic promotion of photomorphogenesis in the *cop1-6pif1* background in the dark and far-red light.

(A-B) (Top) Photographs of cotyledon angles of dark and FRc light grown seedlings, including wild type, *pif1*, *cop1-6*, *cop1-6pif1*, *cop1-6pif1hfr1*, *cop1-6hfr1*, *hfr1pif1* and *hfr1*. Seedlings were grown either in the dark for 5 days (A) or grown in the dark for 21 hours then transferred to continuous FRc (0.06 μmol/m²/s) for 4 days (B). (Bottom) Bar graph showing cotyledon angles of various genotypes as indicated. (C-D) (Top) Photographs of cotyledon areas of dark and FRc light grown seedlings. (Bottom) Bar graph showing cotyledon areas of various genotypes as indicated above. Error bars indicate standard deviation. The letters “A” to “E” indicate statistically significant differences between means for hypocotyl lengths, cotyledon angle and cotyledon area of the indicated genotypes, (p<0.05), (n>30, three biological replicates).
Supplemental Figure 8: HFR1 promotes seed germination under far-red light

(A) Line graph shows the percent of seeds germinated for various genotypes as indicated in the dark and an increasing amount of far-red light intensities. Same stage seeds of Col-0, pif1, hfr1, hfr1pif1, cop1-4, cop1-4hfr1 and cop1-4pif1 were surface sterilized within 1 hour of imbibition and plated on the MS plates. They were exposed to far-red light (34 μmol/m²/s) for 5 mins before being kept in the dark for 48 hours. The seeds were then either kept in the dark continuously or treated with increasing amount of far-red light as indicated and then wrapped again to keep in the dark for 6 additional days before being quantified. The error bars indicate standard deviation (n=40, three biological repeats). (B) The bar graph shows the increased expression of PIF1 direct target genes in the hfr1 mutant seeds compared with wild type Col-0 seeds both under dark and far-red light conditions. Seeds of Col-0 and hfr1 mutant were plated on MS plates supplemented with 100 μM paclobutrazol within 1 hour. Then they were exposed to far-red light (34 μmol/m²/s) for 5 min and kept in the dark for 48 hours. Total RNA was isolated from either 48 hours old dark-grown seeds or 48 hours old dark-grown seeds exposed to far-red light (100 μmol/m²) for 1 hour. Error bars indicate standard deviation (n= 3 independent biological repeats).
Supplemental Figure 9: Model showing the reciprocal degradation of PIF1 and HFR1 by COP1 during the transition from skotomorphogenesis to photomorphogenesis.

(Left) PIF1, COP1, SPA1 and HFR1 directly interact with each other to form a complex. PIF1 promotes the COP1-mediated ubiquitination and subsequent degradation of HFR1 through the 26S proteasome-mediated pathway. HFR1, in one hand, suppresses the transcriptional activity by blocking the DNA binding ability of PIF1; on the other hand, also promotes the PIF1 ubiquitination and degradation by the 26S proteasome pathway. (Right) Under light, the active Pfr form of phytochrome migrates into the nucleus and inhibits the COP1/SPA complex. This results in increased abundance of HFR1, which inhibits PIF1 function to promote seed germination and seedling de-etiolation. PIF1 is also degraded under light resulting in inhibition of PIF1 activated gene expression.
Table S1

Supplemental Table 1: Primer sequences used in experiments described in the text.

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Development 144: doi:10.1242/dev.146936: Supplementary information