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RESEARCH ARTICLE

Molecular bases for the constitutive photomorphogenic phenotypes in *Arabidopsis*

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ABSTRACT

The transition from skotomorphogenesis to photomorphogenesis is regulated in part by the COP1/SPA complex and phytochrome-interacting factors (PIFs) in *Arabidopsis*. The constitutive photomorphogenic (cop) phenotypes of *cop1* and *spaQ* mutants have been shown to result from a high abundance of positively acting transcription factors. Here, we show that the four major PIF proteins are unstable in *cop1* mutants and that overexpression of *PIF1*, *PIF3*, *PIF4* and *PIF5* suppresses *cop1* phenotypes in the dark. A comparison of the transcriptome data among *cop1*, *spaQ* and *pifQ* reveals remarkably overlapping gene expression profiles with preferential regulation of PIF direct target genes. Additionally, HFR1 strongly inhibits the *in vivo* binding and transcriptional activation activity of PIF1 in the dark. Taken together, these data suggest that the cop phenotypes of the *cop1* and *spaQ* mutants are due to a combination of the reduced level of PIFs, increased levels of positively acting transcription factors (e.g. HY5/HFR1) and the HFR1-mediated inhibition of PIF-targeted gene expression in the dark.

This article has an associated 'The people behind the papers' interview.

KEY WORDS: E3 ubiquitin ligase, Phytochrome-interacting factors, Photomorphogenesis, 26S proteasome degradation, Skotomorphogenesis, *Arabidopsis*

INTRODUCTION

Plants have evolved contrasting developmental programs for the successful establishment of young postgermination seedlings early in their life cycle. In darkness, plants undergo skotomorphogenesis, which is defined by elongated hypocotyls, an apical hook and closed cotyledons. This developmental program is suited for protection of the apical region during rapid emergence of seedlings through the soil surface. Once the seedlings are exposed to ambient light, they undergo photomorphogenesis, defined by short hypocotyls, absence of an apical hook, and open, expanded, green cotyledons. This growth pattern allows seedling body plan formation for maximal light capture and autotrophic growth (Gommers and Monte, 2018). Photomorphogenesis has been proposed to be the default pathway for plant development because a series of mutants displaying constitutive photomorphogenic (cop) phenotypes in the dark has been described (Xu et al., 2015). These include 11 loci encoding the *CONSTITUTIVE PHOTOMORPHOGENIC1*/*DE-ETIOLATED1*/*FUSCA* (*COP*/*DET*/*FUS*) genes (Lau and Deng, 2012), four loci encoding *SUPPRESSOR OF PHYA-105* (*SPA1-SPA4*) (Laubinger et al., 2004) and a small family of basic helix-loop-helix (bHLH) transcription factor genes called *PHYTOCHROME-INTERACTING FACTORs* (*PIF1-PIF8*) (Leivar and Quail, 2011; Pham et al., 2018b). These genes encode proteins that act additively and/or synergistically to prevent precocious germination and seedling establishment in the dark.

Among these repressors of photomorphogenesis, COP1 functions as an E3 ubiquitin ligase in association with SPA1-SPA4, targeting a variety of substrates, including the positively acting transcription factors (e.g. HY5/HFR1/LAF1 and others) in light-signaling pathways for Ubiquitin/26S proteasome-mediated degradation (Hardtke et al., 2000; Hoecker, 2017; Jang et al., 2005; Osterlund et al., 2000; Seo et al., 2003; Yang et al., 2005b). COP1, SPAs and CUL4 form $CUL4^{COP1\text{-}SPA}$ E3 ubiquitin ligase complexes that target positively acting factors in the dark (Chen et al., 2010). Consistently, *cop1*, *spaQ* and *cul4cs* (co-suppressed) lines display constitutive photomorphogenic (cop) phenotypes in the dark. In addition, another complex, called the COP9 signalosome (CSN), comprises eight distinct subunits (CSN1-CSN8) and is involved in deconjugation of NEDD8/RUB1 to CULLIN RING ligases (CRLs) (Lau and Deng, 2012; Serino and Deng, 2003). Plants with mutations in any of these subunits also display cop phenotypes in the dark.

DET1 is a nuclear protein that binds to the N-terminal tail of histone H2B and regulates cell type-specific expression of light-regulated genes (Benvenuto et al., 2002; Pepper et al., 1994). It also promotes skotomorphogenesis, in part by stabilizing PIFs in the dark (Dong et al., 2014). In addition, DET1 suppresses seed germination by destabilizing HFR1 and stabilizing PIF1 (Shi et al., 2015). It also interacts with COP10 and DAMAGED DNA-BINDING PROTEIN 1 (DDB1) to form the $CUL4^{CDD}$ complex, which represses photomorphogenesis in the dark, in part by degrading positively acting transcription factors (Chen et al., 2006; Schroeder et al., 2002).

PIFs belong to the bHLH family of transcription factors that repress photomorphogenesis in the dark by promoting skotomorphogenic development. There are eight PIFs (PIF1-PIF8) in *Arabidopsis*, with a high degree of sequence similarity (Pham et al., 2018b). However, individual *pif* mutants display distinct phenotypes, which are especially pronounced in the four major *pif* mutants [*pif1*, *pif3*, *pif4* and *pif5*, collectively called the 'PIF quartet' (*pifQ*)]. For example, *pif1* seeds germinate under red and far-red light as well as in darkness (Oh et al., 2004; Shen et al., 2005), suggesting that PIF1 is a repressor of light-induced seed germination. Both *pif1* and *pif3* mutants have more chlorophyll and carotenoids compared with wild type during the transition from dark to light (Huq et al., 2004; Moon et al., 2008; Stephenson et al., 2009; Toledo-Ortíz et al., 2010), suggesting that PIF1 and PIF3

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suppress the biosynthesis of these pigments. *pif3*, *pif4* and *pif5* mutants display hypersensitive phenotypes in response to red light, in part by inducing co-degradation of these PIFs with phyB (Huq and Quail, 2002; Khanna et al., 2007; Monte et al., 2004; Zhu and Huq, 2014). In this process, multiple kinases (e.g. PPKs) and E3 ubiquitin ligases (e.g. $CUL3^{LRB}$) participate in inducing the co-degradation of PIFs and phyB in response to light (Ni et al., 2017, 2014; Pham et al., 2018b). Thus, phyB is more abundant in these mutants, resulting in hypersensitive phenotypes under red light. In addition, other kinases (e.g. PPKs, BIN2 and CK2) and E3 ubiquitin ligases (e.g. $CUL1^{EBF1/2}$, $CUL1^{CTG10}$, $CUL3^{BOP}$ and $CUL4^{COP1\text{-}SPA}$) induce the degradation of PIFs in response to light in a phytochrome-dependent manner to promote photomorphogenesis (Bernardo-García et al., 2014; Bu et al., 2011; Dong et al., 2017; Majee et al., 2018; Ni et al., 2017; Pham et al., 2018b; Zhang et al., 2017). Strikingly, the quadruple mutant of the PIF quartet, *pifQ*, displays constitutive photomorphogenesis in the dark (Leivar et al., 2008; Shin et al., 2009), suggesting that these PIFs repress photomorphogenesis in the dark. They do so by regulating gene expression directly and indirectly in an individual to a shared manner (Pfeiffer et al., 2014; Pham et al., 2018b).

The cop phenotypes of the *cop1* and *spaQ* mutants were thought to be due primarily to a high abundance of the positively acting transcription factors (e.g. HY5/HFR1/LAF1 and others) in the dark (Hoecker, 2017). However, a few reports showed that PIFs are less abundant in *cop1* mutants (Bauer et al., 2004; Pham et al., 2018a; Shen et al., 2008; Xu et al., 2017; Zhu et al., 2015) and also to a lesser extent in *spaQ* mutants (Leivar et al., 2008; Ni et al., 2014; Pham et al., 2018a), suggesting that the instability of PIFs contributes to the cop phenotypes of these mutants. Here, we show that the gene expression signature of *cop1* and *spaQ* overlaps with *pifQ* in the dark, with preferential targeting of PIF direct target genes, suggesting that the cop phenotype of *cop1* and *spaQ* is partly due to a reduced level of PIFs in these backgrounds. In addition, we also show that the positively acting transcription factor HFR1 strongly inhibits the DNA-binding activity of PIF1 by sequestration; thereby promoting the cop phenotypes of *cop1* and *spaQ* in the dark.

RESULTS

COP1 and SPA positively regulate PIF protein level in darkness

The cop phenotypes of *cop1-4*, *spaQ* and *pifQ* have been previously described (Fig. 1A) (Deng et al., 1992; Laubinger et al., 2004; Leivar et al., 2008; Shin et al., 2009). To examine whether the cop phenotype of *pifQ* is due to a reduction in the COP1 level, we performed immunoblots using an anti-COP1 antibody for 4-day-old dark-grown seedlings of wild type, *cop1-4*, *spaQ* and *pifQ*. Results showed that the COP1 level in *pifQ* and *spaQ* was similar to that in wild-type seedlings (Fig. 1B), suggesting that the *pifQ* phenotype is not due to a reduction in the COP1 level.

Previously, we and others showed that the PIF levels in *cop1* and *spaQ* mutants are reduced compared with wild type in the dark (Bauer et al., 2004; Leivar et al., 2008; Pham et al., 2018a; Zhu et al., 2015). To systematically analyze PIF levels without the transcriptional regulation in these mutants, we crossed the overexpression lines of tagged *PIF1*, *PIF3*, *PIF4* and *PIF5* using the constitutively active 35S promoter in *cop1-4*, TAP-PIF1 and PIF5-Myc in *spaQ* mutant backgrounds, and performed immunoblots for protein levels. Although, the overexpression data might be quantitatively different compared with the native PIF levels in these backgrounds because of high expression, the results showed that all four PIF levels were reduced in *cop1-4*, as previously reported (Fig. 1C-F) (Bauer et al., 2004; Pham et al., 2018a; Xu et al., 2017; Zhu et al., 2015). Both TAP-PIF1 and PIF5-Myc levels were reduced in *spaQ* compared with wild type (Fig. S1). Thus, the cop phenotype of *cop1-4* and *spaQ* might be due, in part, to a reduction in the PIF levels in these backgrounds.

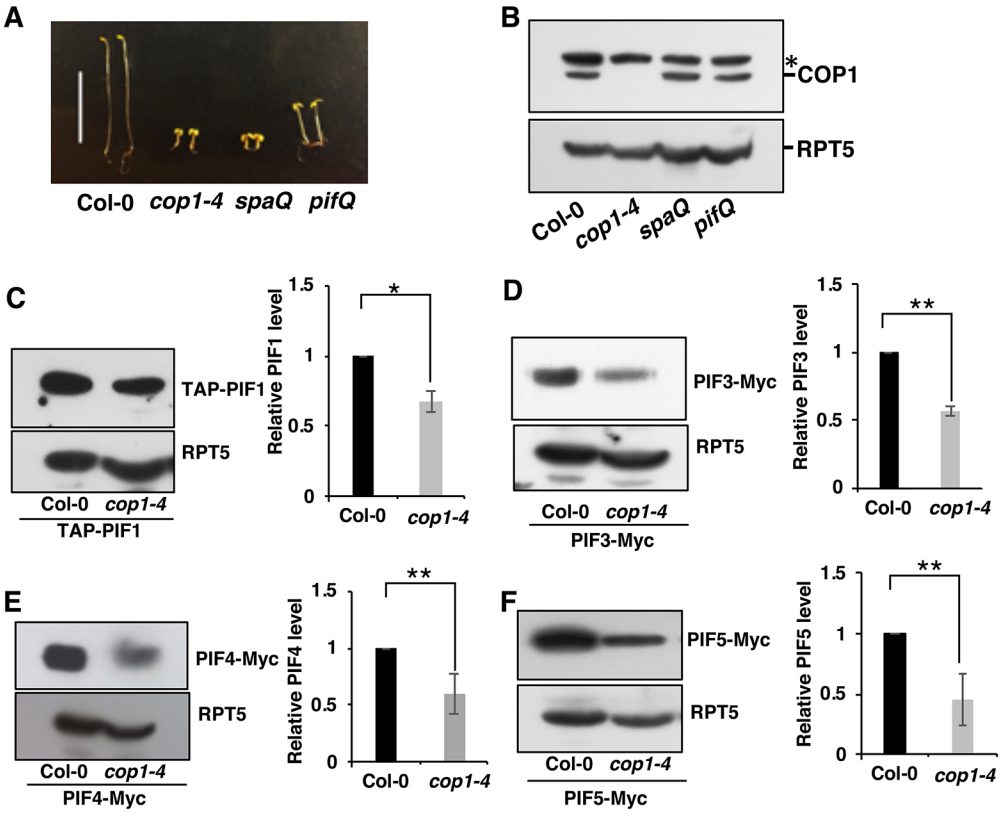

Fig. 1. COP1 positively regulates PIF protein level in darkness. (A) Visible constitutive photomorphogenic phenotypes of 4-day-old dark-grown seedlings. (B) Immunoblot showing COP1 endogenous protein levels in wild-type (Col-0), *cop1-4*, *spaQ* and *pifQ* dark-grown seedlings. Total protein was extracted from 4-day-old dark-grown seedlings, separated on 8% SDS-PAGE gel, and probed with anti-COP1 and anti-RPT5 antibodies. Asterisk indicates a cross-reacting band. (C-F) Immunoblots and graphs showing PIF protein levels. The plants overexpressing *TAP-PIF1*, *PIF3-Myc*, *PIF4-Myc* or *PIF5-Myc* in a Col-0 or *cop1-4* background were grown under the conditions described in the Materials and Methods. Total protein was separated on a 6.5% SDS-PAGE gel and probed with anti-Myc and anti-RPT5 antibodies. PIF protein levels were quantified from three biological replicates (n=3) and normalized with RPT5 levels. The PIF protein level in Col-0 was set as 1. *P<0.05, **P<0.01. Scale bar: 10 mm in A.

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We also examined the transcript levels of the native *PIF*s in the *cop1-4* and *spaQ* mutants using quantitative RT-PCR assays (RT-qPCR; Fig. S2). The transcript levels of *PIF1*, *PIF3* and *PIF4* were similar between Columbia-0 (Col-0) plants and *cop1-4* mutants, whereas the transcript level of *PIF5* was strongly increased in *cop1-4* mutants. In *spaQ* mutants, the transcript levels of *PIF1*, *PIF3* and *PIF4* were slightly lower, whereas the level of the *PIF5* transcript was slightly higher compared with wild-type seedlings. These data illustrate that COP1 and SPA proteins positively regulate PIF protein levels in darkness, possibly by destabilizing HFR1, given that the latter has been shown to induce degradation of PIF1 by heterodimerization (Xu et al., 2017).

PIFs are degraded in *cop1-4* and *spaQ* mutants through the 26S proteasome

PIFs are stable in the dark and undergo degradation in light through the 26S proteasome pathway (Pham et al., 2018b). However, a recent study showed that PIF1 is also degraded in the dark by direct heterodimerization with HFR1 (Xu et al., 2017). To test whether the degradation of other PIFs in the *cop1-4* and *spaQ* backgrounds in the dark is also through the 26S proteasome pathway, we treated dark-grown seedlings with a proteasome inhibitor (bortezomib) for 4 h and then extracted total protein for immunoblots. Results showed that the proteasome inhibitor prevented the degradation of all four PIFs in the *cop1-4* background (Fig. 2). Both TAP-PIF1 and PIF5-Myc were also stabilized in the *spaQ* background upon bortezomib treatment (Fig. S1). These data suggest that the COP1/SPA complex stabilizes PIFs in the dark, probably by destabilizing HFR1.

PIF overexpression partially suppresses the cop phenotypes of *cop1-4* and *spaQ*

If the reduced level of PIFs in the *cop1-4* and *spaQ* backgrounds contributes to the cop phenotypes of these mutants, we hypothesized that an overexpression of these *PIFs* in the *cop1* and *spaQ* mutants is expected to suppress the cop phenotypes. To test this hypothesis, we overexpressed four PIFs (*TAP-PIF1*, *PIF3-Myc*, *PIF4-Myc* and *PIF5-Myc*) in the *cop1-4* background and two PIFs (*TAP-PIF1* and *PIF5-Myc*) in the *spaQ* background and examined their phenotypes in the dark. Results showed that, whereas the hypocotyl lengths of TAP-PIF1/*cop1-4* and PIF3-Myc/*cop1-4* were comparable to those of *cop1-4*, the hypocotyl lengths of PIF4-Myc/*cop1-4* and PIF5-Myc/*cop1-4* were significantly longer compared with *cop1-4* (Fig. 3A-C). Moreover, all four *PIF* overexpression lines in the *cop1-4* mutant displayed a significantly smaller cotyledon opening angle compared with that of *cop1-4* (Fig. 3B,D), suggesting that PIFs suppress the cop phenotypes of *cop1-4*. Similarly, an overexpression of *PIF5-Myc* in the *spaQ* background suppressed both the hypocotyl lengths and cotyledon angle phenotypes of the *spaQ* mutant compared with overexpression of *spaQ* only, whereas overexpression of *TAP-PIF1* in *spaQ* only suppressed the cotyledon angle phenotype (Fig. S3A-C). These data also suggested that the cop phenotype of *cop1-4* and *spaQ* is partially due to a reduced level of PIFs.

cop1-4, *spaQ* and *pifQ* display a large overlapping set of co-regulated genes

Previously, the cop phenotypes of the *cop1* mutant were demonstrated to mainly result from the high abundance of positively acting transcription factors (e.g. HY5/HFR1/LAF1 and others) in the dark (Hoecker, 2017). Given that PIFs were unstable in *cop1* and *spaQ* mutants in the dark (Fig. 1) (Bauer et al., 2004; Leivar et al., 2008; Pham et al., 2018a; Zhu et al., 2015), we hypothesized that COP1-, SPA- and PIF-regulated genes might overlap in genome-wide expression analyses. To test this hypothesis, we analyzed the results from previously published data (Zhang et al., 2013), and our own recent RNA-sequencing (RNA-Seq) experiments using *cop1-4*, *spaQ* and *pifQ* mutant seedlings grown in darkness. Although these experiments were performed in two different laboratories and the growth conditions were slightly different, the results showed that a large proportion of the differentially expressed genes (1120) overlapped among *cop1-4*, *spaQ* and *pifQ* (Fig. 4A,B; Data set S1). Approximately 39% of the PIF-regulated genes displayed overlapping expression patterns with COP1- and SPA-regulated

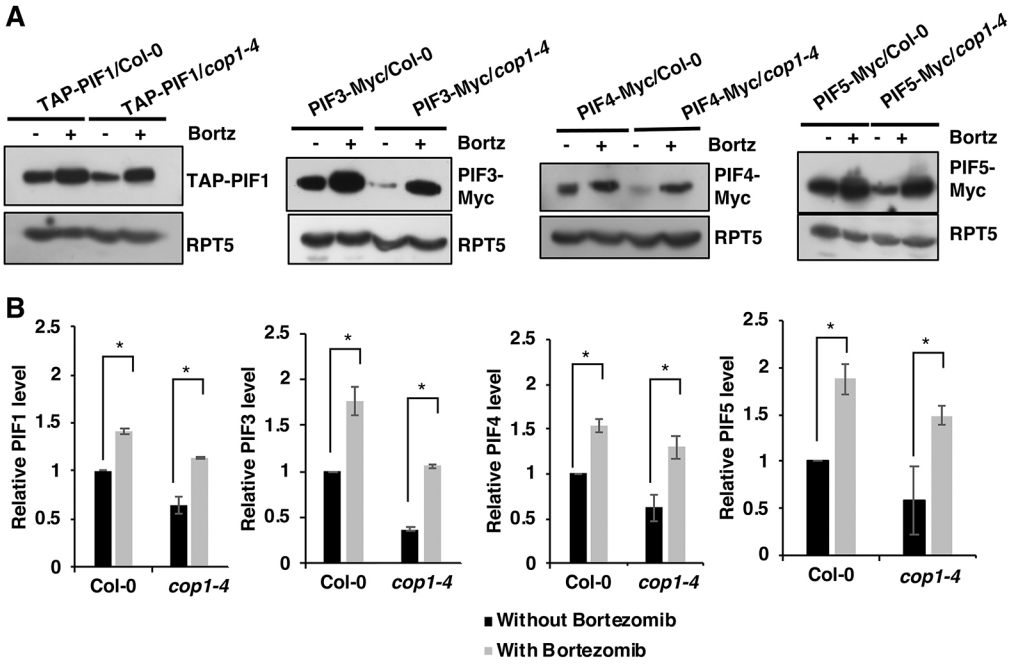

Fig. 2. Instability of PIFs in the dark in *cop1* backgrounds is 26S-proteasome dependent. (A) PIF protein levels in 4-day-old dark-grown wild-type and *cop1-4* seedlings with and without treatment with a 26S protease inhibitor (bortezomib or Bortz). Total proteins were extracted and separated on 6.5% SDS-PAGE gels and probed with anti-Myc and anti-RPT5 antibodies. RPT5 was used as a loading control. (B) Bar graphs showing the quantitative PIF protein levels in those backgrounds from three biological replicates (n=3). In each graph, the PIF protein level in the Col-0 background without bortezomib treatment was set as 1. Data are mean±s.d. *P<0.05.

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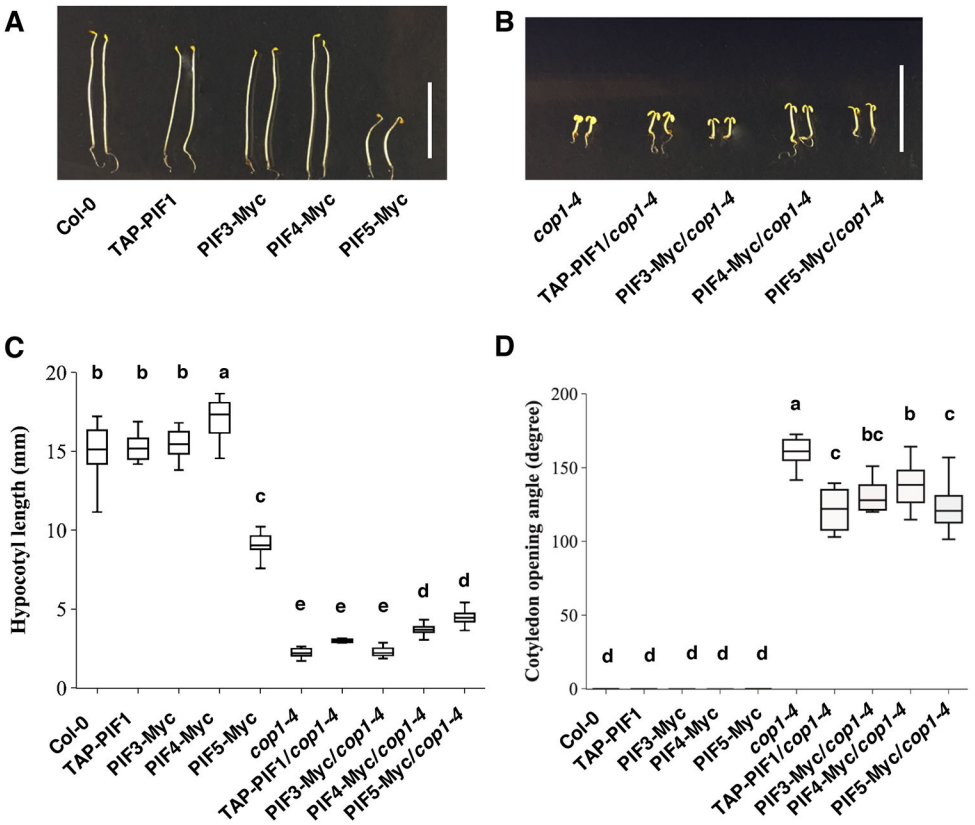

Fig. 3. Overexpression of PIFs partially suppresses the constitutive photomorphogenetic phenotypes of *cop1-4*. (A,B) Visible phenotypes of 4-day-old dark-grown seedlings with *PIF* overexpression in Col-0 and *cop1-4* backgrounds. (C,D) Box plots representing the hypocotyl lengths and cotyledon opening angle measurements. Three independent biological replicates were performed with an average of 30 Col-0 or *cop1-4* seedlings with *PIF* overexpression grown under the same conditions as described in the Materials and Methods. Significant differences between the different genotypes were determined using one-way ANOVA and Tukey's HSD tests, indicated by different letters. Scale bar: 10 mm.

genes. Among these 1120 genes, 483 genes were upregulated and 431 genes were downregulated in all three backgrounds compared with wild type (Fig. 5A,B). Interestingly, 206 of the PIF-regulated genes displayed opposite regulation to the COP1- and SPA-regulated genes (Fig. 4A,B; Data set S3).

To identify the biological processes controlled by these co-regulated genes, we performed Gene Ontology (GO) analyses of the co-regulated genes and divided them into two classes: upregulated versus downregulated genes (Fig. 4C,D; Data set S2). A total of 94 enriched GO terms were identified for these co-regulated genes (Fig. S4). The co-upregulated genes were enriched in chlorophyll biosynthetic processes, defense responses, photosynthesis, response to light stimulus (including red light and blue light), response to cold, and cytokinin. The co-downregulated genes were involved in the regulation of transcription, cell wall organization, response to hormones (abscisic acid and auxin), response to red light, and also metabolic processes. These results were consistent with the cop phenotypes of these mutants.

We performed further analysis on the PIF-regulated 206 genes that displayed opposite regulation by COP1 and SPA (Fig. 4A,B; Data set S3). GO analyses of these 206 genes oppositely regulated between *pifQ* and *cop1-4*/*spaQ* using Database for Annotation, Visualization and Integrated Discovery (DAVID) (Data set S3) and GO Analysis Toolkit and Database for Agricultural Community (AgriGo) (Figs S5 and S6) showed that PIFs function oppositely compared with COP1 and SPA proteins in a few biological processes. For example, many genes involved in responses to UV-B and flavonoid biosynthesis were downregulated in *pifQ*, but upregulated in *cop1-4* and *spaQ* (Fig. S5; Data set S3). Similarly, many other genes involved in defense responses, salicylic acid (SA) metabolism and signaling were upregulated in *pifQ*, but downregulated in *cop1-4* and *spaQ* (Fig. S6; Data set S3. Thus, although PIFs and the COP/SPA complex repress photomorphogenesis coordinately, they also function antagonistically in a few biological processes.

Direct target genes of PIFs are co-regulated in *cop1-4* and *spaQ*

If COP1 and SPA repress photomorphogenesis in the dark, in part by stabilizing PIFs, then the PIF direct target gene expression is expected to be affected in *cop1-4* and *spaQ*. Interestingly, among 338 PIF direct target genes (Pfeiffer et al., 2014), 170 (>50%) genes were co-regulated by *cop1-4* and *spaQ* (Fig. 5A; Data set S4). Furthermore, among the 209 PIF-induced genes, 110 genes were downregulated in *cop1-4* and *spaQ*. In addition, among 129 PIF-repressed genes, 42 genes were upregulated in the *cop1-4* and *spaQ* backgrounds (Fig. 5A; Data set S4). GO analyses revealed that most of these genes function in response to red and far-red light signaling, auxin responses, and the regulation of transcription. Strikingly, the degree and direction of expression of these PIF direct genes were similar among all three cop mutant groups.

To verify the RNA-Seq data by an independent method, we selected a subset of PIF direct target genes involved in auxin responses, cell wall organization and photosynthesis, and performed RT-qPCR analyses to determine the relative expression patterns in the *cop1-4*, *spaQ* and *pifQ* mutants compared with wild type. Results showed a strikingly similar pattern among *cop1-4*, *spaQ* and *pifQ* for both PIF-induced and -repressed genes (Fig. 5D,E), consistent with the RNA-Seq data (Fig. 5B,C). These data also suggested that the *cop* phenotype of the *cop1* and *spaQ* mutants is partly due to the reduced level of PIFs and their target gene expression.

HFR1 represses the transcriptional activity of PIF1

Previously, it was shown that HFR1, a HLH transcription factor, is more abundant in the *cop1-4* and *spaQ* backgrounds compared with wild type (Hoecker, 2017). Given that HFR1 inhibits the

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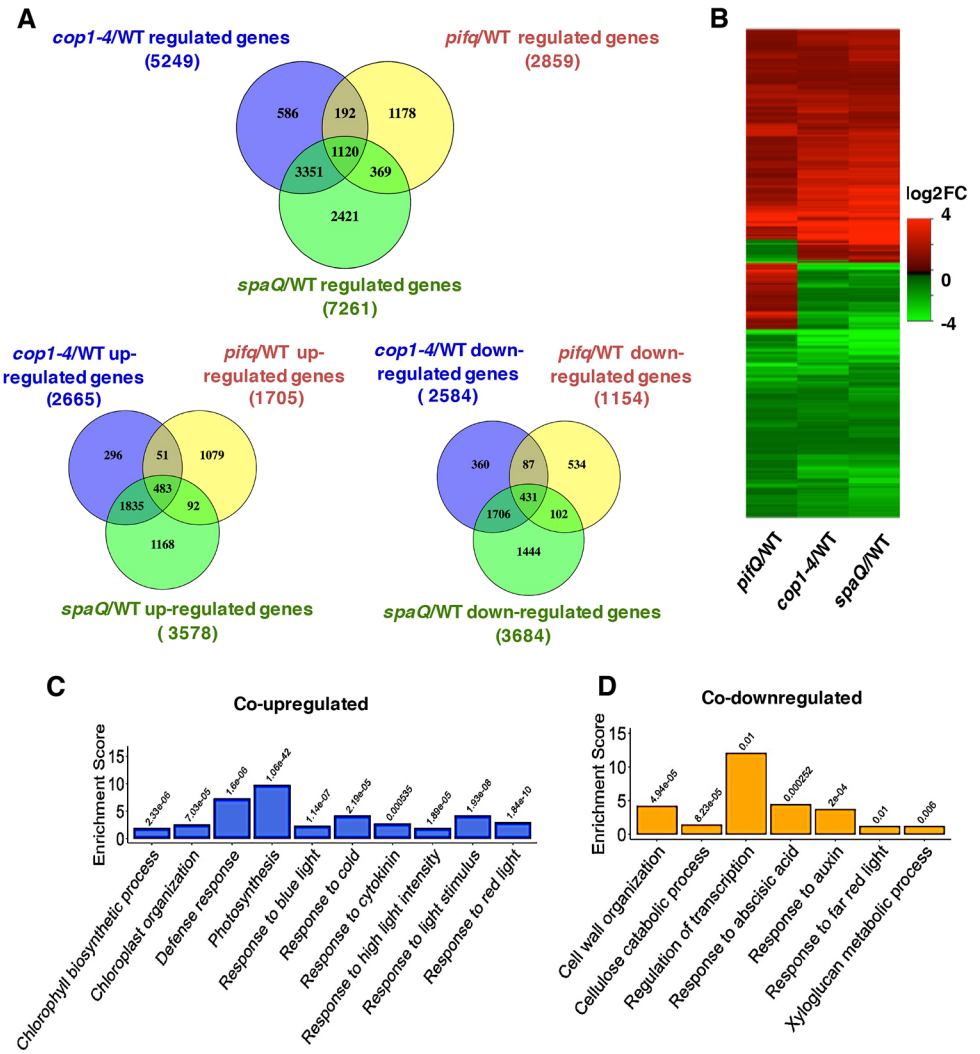

Fig. 4. COP1 and SPA mediate light-regulated transcriptomic changes partly through PIFs. (A) Venn diagram showing 1120 co-regulated, 483 co-upregulated and 431 co-downregulated differentially expressed genes in three different pairwise comparisons (*cop1-4*/WT, *spaQ*/WT and *pifQ*/WT). (B) Hierarchical clustering displaying 1120 differentially expressed genes in comparisons indicated. The data show co-regulated genes identified as having at least a 2-fold difference in gene expression (FDR<0.05). The color represents the log2 of the -fold change in expression. *cop1-4*/WT and *spaQ*/WT: comparison of the expression profiles of dark-grown *cop1-4* and *spaQ* with Col-0, respectively. (C,D) Bar graphs showing enrichment analysis of GO biological processes significantly co-upregulated (C) and co-downregulated (D) in *cop1-4*/WT, *spaQ*/WT, and *pifQ*/WT seedlings. Enrichment scores indicate the percentages of involved genes/total genes. Fisher exact *P*-values are presented on the top of each bar.

DNA-binding activity of PIFs (Shi et al., 2013) and that the PIF levels were reduced in the *cop1-4* and *spaQ* mutants (Fig. 1B-F; Fig. S1A), we hypothesized that the high abundance of HFR1 in the *cop1-4* and *spaQ* backgrounds might contribute to the cop phenotypes of these mutants. To test this hypothesis, we selected a subset of the PIF1 direct target genes that are also regulated by HFR1 and performed RT-qPCR analyses using dark-grown wild-type Col-0, *pifQ*, *cop1-4* and *cop1-4hfr1* seedlings. Results showed that the selected genes were expressed at a reduced level in both *pifQ* and *cop1-4* mutant backgrounds, similar to the RNA-Seq data (Fig. 6A). Strikingly, the expression level of these genes was higher in the *cop1-4hfr1* double-mutant background compared with *cop1-4* (Fig. 6A). However, the increased expression of the PIF1 target genes in the *cop1-4hfr1* mutant might be due to either the high PIF1 protein level and/or the loss of suppression by HFR1 of the DNA-binding activity of PIF1. To distinguish between these possibilities, we performed chromatin immunoprecipitation followed by qPCR (ChIP-qPCR) for dark-grown seedlings expressing the TAP-PIF1 fusion protein in the *cop1-4* and *cop1-4hfr1* backgrounds. We also examined the immunoprecipitated TAP-PIF1 protein level in all these backgrounds during the ChIP assay (Fig. 6B, inset), and divided the promoter enrichments by the protein levels for each genotype to calculate the relative promoter occupancy of PIF1 independent of PIF1 protein level. Results showed that the immunoprecipitated TAP-PIF1 protein level was lower in the *cop1-4* background, but higher in the *cop1-4hfr1* double-mutant background compared with the TAP-PIF1-only background, as previously reported (Fig. 6B, inset) (Zhu et al., 2015). The relative promoter occupancy of PIF1 showed that the DNA-binding activity of TAP-PIF1 was also reduced in the *cop1-4* background and increased in the *cop1-4hfr1* background (Fig. 6B). These data further extend the recent report that HFR1 suppresses the DNA-binding activity of PIF1 not only in imbibed seeds (Shi et al., 2013), but also in seedlings. Thus, HFR1 not only regulates the protein abundance, but also the DNA-binding activity of PIF1 in etiolated seedlings.

To examine the significance of regulation of PIF1 by HFR1, we measured the hypocotyl lengths of dark-grown of wild-type, *cop1-4*, *cop1-4hfr1*, *cop1-4*/*TAP-PIF1*, *cop1-4hfr1*/*TAP-PIF1* and *TAP-PIF1* seedlings. Results showed that the hypocotyl lengths of *cop1-4hfr1*/*TAP-PIF1* seedlings were longer than that of *cop1-4hfr1* seedlings (Fig. 6C,D). The hypocotyl length of *cop1-4* seedlings was similar to that of *cop1-4*/*TAP-PIF1* seedlings, possibly because of the reduced TAP-PIF1 protein level and/or increased sequestration of TAP-PIF1 by HFR1 in the *cop1-4* background. Thus, TAP-PIF1 has an increased function in regulating hypocotyl lengths in the *cop1-4hfr1* background compared with the *cop1-4*-only background.

DISCUSSION

Analyses of cop mutants have had an important role in our understanding of light-signaling pathways in plants. The prevailing

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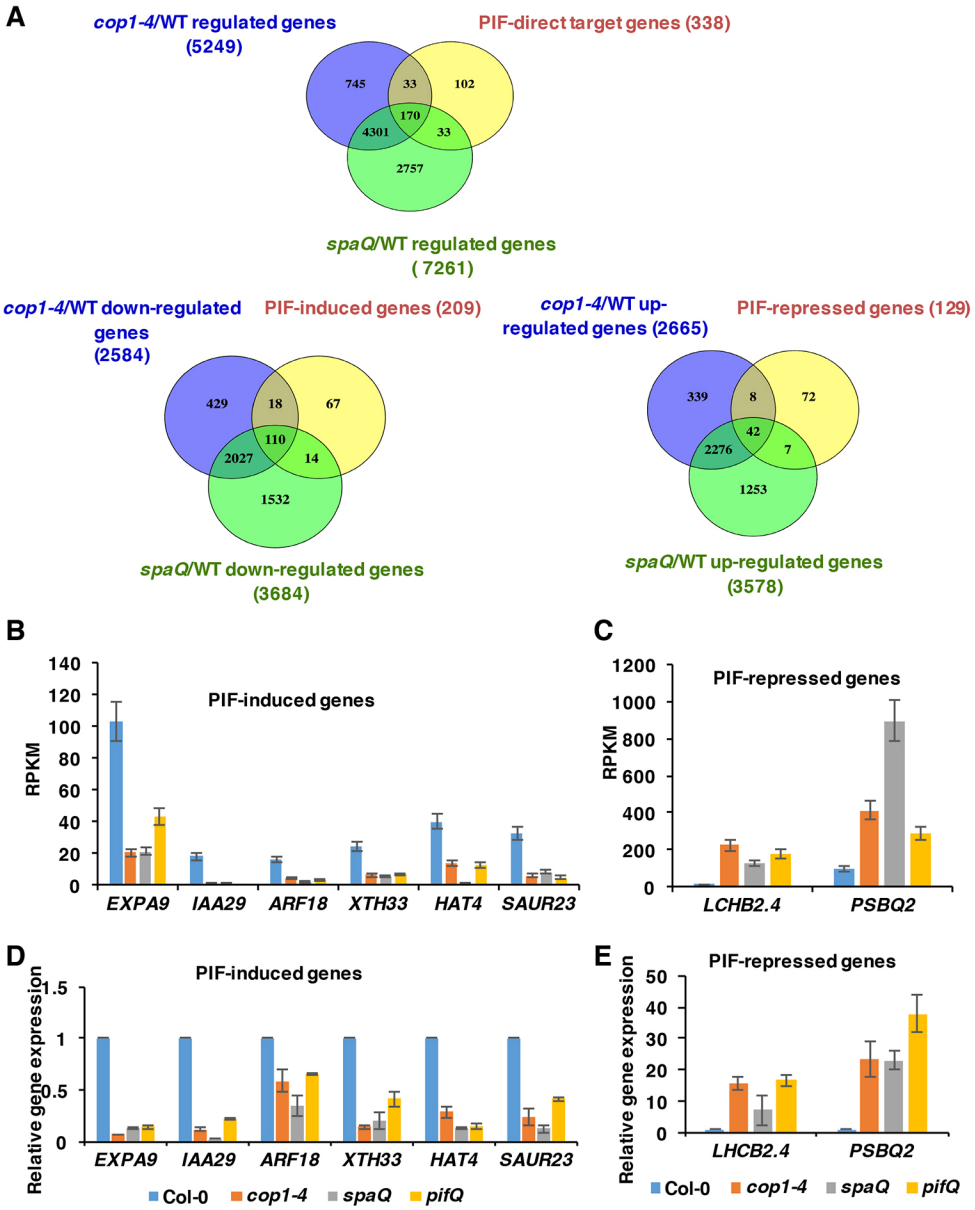

Fig. 5. A significant number of PIF-direct target genes is co-regulated in *cop1-4* and *spaQ* mutants. (A) Venn diagram showing that, among 338 PIF direct target genes, 170 genes are co-regulated by COP1 and SPA, 110 genes are downregulated and 42 genes are upregulated in *cop1-4* and *spaQ* mutants. (B,C) RNA-Seq expression patterns of various PIF-induced genes (B) and PIF-repressed genes (C) in *cop1-4*, *spaQ* and *pifQ* seedlings grown in the dark. (D,E) qRT-PCR shows the similar expression patterns of various PIF-induced genes (D) and PIF-repressed genes (E) in *cop1-4*, *spaQ* and *pifQ* seedlings grown in the dark. Gene expression levels in mutants were normalized to *PP2A* and the expression level in Col-0 was set as 1.

view of the molecular basis of the cop phenotype is that the increased abundance of the positively acting transcription factors (e.g. HY5/HFR1/LAF1 and others) in *cop1-4* and *spaQ* mutants in the dark results in cop phenotypes under darkness (Jang et al., 2005; Osterlund et al., 2000; Saijo et al., 2003; Seo et al., 2003; Yang et al., 2005a,b). Although several studies have reported a reduced abundance of PIFs in various cop mutants compared with wild type (Bauer et al., 2004; Leivar et al., 2008; Ni et al., 2014; Pham et al., 2018a; Shen et al., 2008; Zhu et al., 2015), the mechanism of this reduction and its contribution to the cop phenotype remain unknown. Here, we provide biochemical, molecular and genomic evidence supporting the hypothesis that the reduced PIF levels in *cop1* and *spaQ* mutants contribute to their cop phenotypes. First, we showed that PIFs are actively degraded in the dark in the *cop1-4* and *spaQ* backgrounds through the 26S proteasome pathway (Fig. 1, Fig. S1). Second, the genome-wide gene expression patterns largely overlapped among COP1-, SPA- and PIF-regulated genes, with an altered expression of a set of PIF direct target genes (Figs 4 and 5). Third, PIF1 was sequestered in the *cop1-4* background by an increased abundance of HFR1 and possibly other HLH proteins, resulting in reduced PIF activity in the *cop1-4* background (Fig. 6). Fourth, overexpression of *PIF1* in the *cop1-4hfr1* background promoted hypocotyl elongation in the dark. Fifth, overexpression of major *PIF*s in the *cop1-4* and *spaQ* backgrounds suppressed the cop phenotypes of the *cop1-4* and *spaQ* mutants (Fig. 3, Fig. S3). Overall, these data suggest that the reduction in PIF levels and PIF activity in the *cop1-4* and *spaQ* backgrounds contributes to their cop phenotypes.

Despite similar morphological and molecular phenotypes among *cop1-4*, *spaQ* and *pifQ*, the GO analyses of the differentially expressed genes oppositely regulated between *pifQ* and *cop1-4*/*spaQ* revealed that these genes also have distinct roles in plant signaling pathways. One of the striking differences is in the enrichment of the genes involved in SA metabolism and signaling in *pifQ* compared with *cop1-4* and *spaQ*, suggesting that PIFs suppress defense responses, as previously discussed (Paik et al., 2017). In fact, PIFs are known to promote growth possibly by suppressing defense responses, given that a trade-off between growth versus

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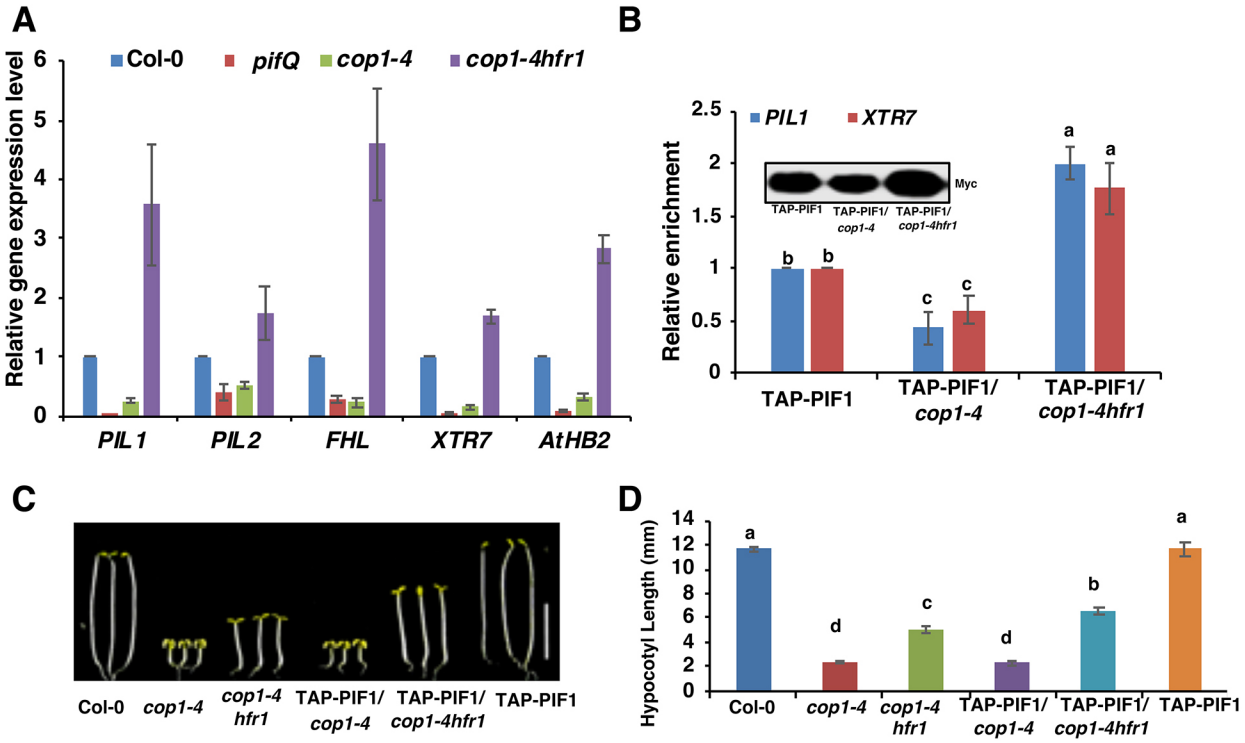

Fig. 6. The transcriptional activation activity of PIFs is higher in the *cop1-4hfr1* background compared with the *cop1-4* background. (A) The expression levels of PIF target genes are lower in the *pifQ* and *cop1-4* backgrounds, but higher in the *cop1-4 hfr1* background. Total seedling RNA was extracted from 3-day-old dark-grown wild-type Col-0, *pifQ*, *cop1-4* and *cop1-4hfr1* seedlings. *PP2A* was used as an internal control. Wild-type Col-0 was set as 1. Error bars indicate standard deviation (n=3 independent biological repeats). (B) The *PIL1* and *XTR7* promoter occupancies of TAP-PIF1 were reduced in the *cop1-4* background but increased in the *cop1-4hfr1* background. ChIP-qPCR assays were performed on 3-day-old dark-grown seedlings expressing TAP-PIF1 fusion protein on *cop1-4* and *cop1-4hfr1* backgrounds. (C) Photographs of wild-type, *cop1-4*, *cop1-4hfr1*, *cop1-4*/TAP-PIF1 and *cop1-4hfr1*/TAP-PIF1 seedlings. Seedlings were grown in the dark for 5 days. (D) Bar graph showing hypocotyl lengths of various genotypes as described in C. Error bars indicate standard deviation. Significant differences between different genotypes were determined using one-way ANOVA and Tukey's HSD tests, indicated by different letters. Scale bar: 5 mm.

defense is a well-known phenomenon in plant growth and development (Paik et al., 2017). By contrast, the genes involved in UV-B responses and flavonoid biosynthesis were downregulated in *pifQ*, but upregulated in *cop1-4* and *spaQ*. Although, a role for PIFs in UV-B signaling has not yet been examined in detail, a recent study suggested that PIFs are involved in UV-B-induced leaf hyponasty (Fierro et al., 2015). However, the COP1 and SPA proteins function positively in UV-B signaling pathways (Huang et al., 2013; Tilbrook et al., 2013). Overall, these analyses highlight both common and distinct functions of PIFs, COP1 and SPA proteins in regulating biological processes in plants.

COP1/SPA proteins might regulate the abundance and activity of PIFs in multiple ways. For example, a recent study showed a noncanonical function of the COP1/SPA complex in inhibiting BIN2 kinase from phosphorylating PIF3 and regulating PIF3 abundance (Ling et al., 2017). Another source of the opposing functions between PIFs and the COP1/SPA complex is the increased abundance of the positively acting transcription factors, especially HFR1, in the *cop1-4* and *spaQ* backgrounds. HFR1 is an atypical bHLH protein that sequestered PIFs from binding to DNA as well as reducing PIF abundance (Fig. 6) (Hornitschek et al., 2009; Shi et al., 2013; Xu et al., 2017). Similar to HFR1, the HECATE family of bHLH proteins also inhibits PIF activity, and is degraded in the dark, possibly by the COP1/SPA complex (Zhu et al., 2016). Thus, the COP1/SPA complex might negatively regulate the abundance of factors that function antagonistically to PIFs.

In summary, we propose a revised model for the molecular bases of cop phenotypes in plants (Fig. 7). First, as previously hypothesized, an increased abundance of the positively acting transcription factors (e.g. HY5, LAF1, HFR1 and others) in the *cop1* and *spaQ* mutants promotes photomorphogenesis in the dark. Second, a reduced level of PIFs in the *cop1* and *spaQ* mutants contributes to the cop phenotype in the dark. Finally, a reduction in PIF activity because of the increased abundance of atypical bHLH proteins (e.g. HFR1, HECATE and possibly others) in *cop1* and *spaQ* mutants additively promotes the cop phenotypes. It is notable that all three activities are tightly linked to each other, contributing in concert to the skotomorphogenic and photomorphogenic development of plants.

MATERIALS AND METHODS

Plant materials, growth conditions and measurements

Seeds of the Col-0 ecotype of *Arabidopsis thaliana* were used for all experiments. Seeds were surface sterilized and then plated on Murashige and Skoog (MS) medium without sucrose. After stratification at 4°C for 3 days, seeds were exposed to white light for 3 h at room temperature to trigger germination before placing them back in the dark for an additional 4 days. These 4-day-old seedlings were then used for protein extraction for western blots and measurements of hypocotyl lengths and cotyledon opening angle phenotypes, using the ImageJ software (rsb.info.nih.gov/ij/). A total of 90 seedlings from three biological replicates were measured. Significant differences between different genotypes were determined using one-way ANOVA and Tukey's HSD tests, indicated by different letters in the figures accompanying this report.

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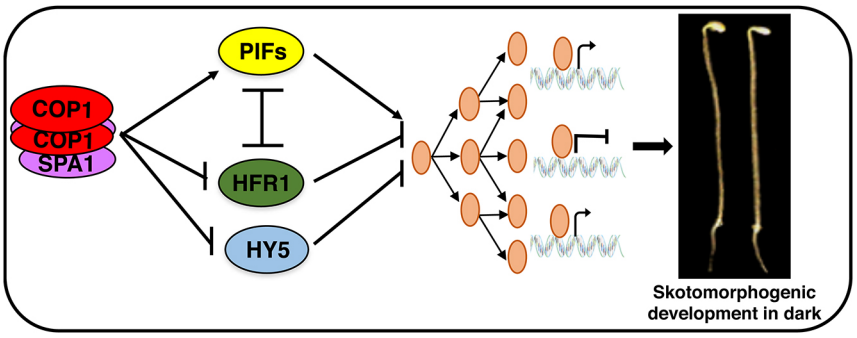

Fig. 7. Model showing how COP1 and SPA proteins regulate various transcription factors to promote skotomorphogenesis in the dark. Mutations in COP1, SPA and PIFs result in cop phenotypes in the dark. PIFs and HFR1 reciprocally regulate their abundance, whereas HFR1 inhibits PIF activity by sequestration. Regulation of the abundance and activity of these transcription factors by the COP1-SPA complex promotes skotomorphogenic development.

Generation of transgenic lines

The *pif1*, *pifQ*, *cop1-4*, *cop1-4hfr1*, *cop1-4*/TAP-PIF1 (Castillon et al., 2009; Xu et al., 2014; Zhu et al., 2015), *TAP-PIF1* (Bu et al., 2011), *PIF3-Myc* (Park et al., 2004), *PIF4-Myc* (Shor et al., 2017) and *PIF5-Myc* (Sakuraba et al., 2014) plants were as previously published. *PIF* overexpression lines were crossed with *cop1-4* and *spaQ* mutants. The crossed homozygous lines were selected from the F3 population using antibiotic selection. The *cop1-4* mutants were selected by sequencing. The *spaQ* homozygous lines were selected by genotyping *spa* mutants. Primers used for sequencing and genotyping are listed in Table S1. For the generation of *cop1-4hfr1*/TAP-PIF1, *cop-4hfr1* was crossed into TAP-PIF1 to obtain the F1 generation. *cop1-4hfr1*/TAP-PIF1 was obtained by genotyping and antibiotic selection (gentamycin) of the F2 and F3 generations. The primers used for sequencing and genotyping are listed in Table S1.

Transcriptomic analyses

RNA-Seq was performed using 3-day-old dark-grown seedlings. Seeds were kept in the dark for 3 days at 4°C and exposed to 3 h of white light. After 21 h in the dark, plates were then treated with 2000 µmol m^{-2} far-red light for the true-dark condition, as previously described (Leivar et al., 2008). Total RNA was extracted after 2 days in darkness. Raw data and processed data for the total read counts of sequencing reads in Col-0, *cop1-4* and *spaQ* can be accessed from the Gene Expression Omnibus database under accession number GSE112662.

For the RNA-seq analysis, raw read quality was accessed using FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/). Raw reads were then aligned to the *Arabidopsis* genome using Bowtie2 (Langmead and Salzberg, 2012) and TopHat (Trapnell et al., 2012). The annotation of the *Arabidopsis* genome was obtained from TAIR10 (www.arabidopsis.org/). Read count data were performed by HTseq (Anders et al., 2015) (htseq.readthedocs.io/en/master/index.html).

Differentially expressed genes in *cop1-4*/WT and *spaQ*/WT were identified using the DESeq2 package (Love et al., 2014). The differential gene expression was defined as those differing by ≥2-fold with adjusted *P* value (FDR) ≤0.05.

Differentially expressed genes in *pifQ* and the PIF differential direct target genes list were obtained from RNA-Seq and ChIP-Seq data, respectively, under the accession number GSE43286 (Pfeiffer et al., 2014). Venn diagrams were generated using Venny 2.1.0 (bioinfogp.cnb.csic.es/tools/venny/). Heatmaps were generated using DESeq2 and the ComplexHeatmap package (Gu et al., 2016) in the R statistical program. GO enrichment analyses were performed using DAVID v6.8 (david.ncifcrf.gov/). GO bar graphs were generated based on the significant enriched terms with the lowest *P* value and FDR (≤0.05) for GO terms. Hieratical graph results for the GO term analysis of *cop1-4*-, *spaQ*- and *pifQ*-regulated genes were also performed by AgriGo (bioinfo.cau.edu.cn/agriGO/index.php).

RT-qPCR assay

For determining the transcript levels of *PIF*s and PIF direct target genes by RT-qPCR assays, total RNA was extracted from seedlings grown under the same conditions used for the RNA-Seq experiments. M-MLV reverse transcriptase (Thermo Fisher Scientific) was used to reverse transcribe 1 µg of total RNA treated with on-column DNase I (Sigma Aldrich). A RT-qPCR assay was performed using Power SYBR green (Applied Biosystems). Gene-specific primers are listed in Table S1. *PP2A* (At1g13320) was used as the internal control to normalize the expression of different genes. The calculation of the levels of expression of different genes relative to *PP2A* was as follows: $2^{\Delta Ct}$, where ΔCt=Ct (*PP2A*)−Ct (specific gene) and Ct indicates the cycle threshold values. Relative expression was quantified from three biological replicates. Error bars indicate mean±s.d. Student's *t*-test assuming unequal variances was performed, and the *P* values are indicated in each figure.

Protein extraction and immunoblot analyses

For examination of the COP1 protein level, 0.2 g tissue from 4-day-old dark-grown seedlings was extracted in extraction buffer as previously described (Zhu et al., 2015). Total protein was separated on 8% SDS-PAGE gels. Proteins were transferred to a PVDF membrane and western blots were detected with anti-COP1 or anti-RPT5 (Enzo Life Sciences) antibodies for endogenous COP1 and RPT5, respectively.

For PIF protein levels in the Col-0 and mutant backgrounds, total protein from 50 seedlings was extracted using 50 µl urea extraction buffer [48% urea (w/v), 0.1 M phosphate buffer pH 6.8, 10 mM Tris-Cl pH 6.8, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1× protease inhibitor cocktail]. Samples were centrifuged at 16,000 ***g*** for 10 min and heated at 65°C for 10 min. Supernatants were analyzed on 6.5% SDS-PAGE gels and detected using anti-Myc (dilution 1/1000, OP10-200UG, EMD Millipore) and anti-RPT5 antibodies (dilution 1/3000, BML-PW8245-0100, Enzo Life Sciences).

For treatment with a 26S proteasome inhibitor (bortezomib, B-1408, LC Laboratories), 4-day-old dark-grown seedlings were transferred to 5 ml liquid MS media containing 40 µM bortezomib and incubated in the dark for 4 h. Total protein was then extracted using the urea extraction buffer as described earlier. For quantitation of protein levels, ImageJ software was used to measure the band intensities from three independent biological replicates, and normalized to RPT5 protein levels. Error bars indicate mean±s.d. Student's *t*-test assuming unequal variances was performed, and *P* values are indicated in each figure.

ChIP assay

ChIP-qPCR assays were performed on 3-day-old dark-grown seedlings expressing TAP-PIF1 fusion protein in *cop1-4* and *cop1-4hfr1* backgrounds. Anti-Myc (71D10, Cell Signaling Technologies) antibody was used to immunoprecipitate TAP-PIF1 and associated DNA. DNA was amplified using primers specific to the G-box fragment or control regions. Anti-Myc (OP10-200UG, EMD Millipore) antibody was used to determine the immunoprecipitated TAP-PIF1 protein level in each background. Both the TAP-PIF1 promoter enrichment from the ChIP-qPCR and TAP-PIF1 protein level quantified by ImageJ were set as 1. The relative enrichment of the -fold change in *cop1-4*/TAP-PIF1 and *cop1-4hfr1*/TAP-PIF1 were first normalized compared with the TAP-PIF1 only for their promoter enrichment and protein levels, respectively, and the promoter enrichment levels were then divided by the protein levels for each repeat. Final averages of three independent biological repeats for each genotype were calculated

and shown as a bar graph (Fig. 6). One biological repeat of the TAP-PIF1 protein level was shown as an example (Fig. 6B, inset). Error bars indicate standard deviation (n=3 independent biological repeats).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: E.H., V.N.P.; Methodology: V.N.P., X.X.; Validation: V.N.P.; Formal analysis: E.H., V.N.P., X.X.; Investigation: V.N.P., X.X.; Writing - original draft: E.H., V.N.P.; Writing - review & editing: E.H., V.N.P., X.X.; Supervision: E.H.; Project administration: E.H.; Funding acquisition: E.H.

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Data availability
Raw data for the RNA-Seq in this study have been deposited in Gene Expression Omnibus (GEO) under accession number GSE112662. RNA-Seq data for PIF-regulated genes and ChIP-Seq data for PIF direct target genes are available in GEO under accession numbers GSE39217 and GSE43286, respectively.

Supplementary information
Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.169870.supplemental

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