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Article type : Original Article

Dynamic regulation of PIF5 by COP1-SPA complex to optimize photomorphogenesis in *Arabidopsis*

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Keywords: bHLH transcription factor, E3 Ubiquitin ligase, photomorphogenesis, Proteasome degradation, photoperiodic hypocotyl growth.

Running title: Dynamic regulation of PIF5 by COP1-SPA complex

ABSTRACT

Light signal provides the spatial and temporal information for plants to adapt to the prevailing environmental conditions. Alterations in light quality and quantity can trigger robust changes in global gene expression. In *Arabidopsis thaliana*, two groups of key factors regulating those changes

in gene expression are CONSTITUTIVE PHOTOMORPHOGENESIS/ DEETIOLATED /FUSCA (COP/DET/FUS) and a subset of basic helix-loop-helix transcription factors called PHYTOCHROME-INTERACTING FACTOR (PIFs)**.** Recently, a rapid progress has been made in characterizing the E3 Ubiquitin ligases for the light-induced degradation of PIF1, PIF3 and PIF4. However, the E3 ligase(s) for PIF5 is still unknown. Here, we show that $CUL4^{COP1\text{-}SPA}$ complex is necessary for the red light-induced degradation of PIF5. Furthermore, COP1 and SPA proteins stabilize PIF5 in the dark, but promote the ubiquitination and degradation of PIF5 in response to red light through the 26S proteasome pathway. Genetic analysis illustrates that overexpression of *PIF5* can partially suppress both *cop1-4* and *spaQ* seedling de-etiolation phenotypes in the dark and red light conditions. In addition, PIF5 protein level cycles under both diurnal and constant light conditions, which is also defective in the *cop1-4* and *spaQ* backgrounds. Both *cop1-4* and *spaQ* show defect in diurnal growth pattern. Overexpression of *PIF5* partially restores the growth defects in the *cop1-4* and *spaQ* under diurnal conditions, suggesting that COP1-SPA complex plays an essential role in photoperiodic hypocotyl growth, partly through regulating PIF5 level. Taken together, our data illustrate how $CUL4^{COP1\text{-}SPA}$ E3 ligase dynamically controls PIF5 level to regulate plant development.

INTRODUCTION

Seedlings experience two contrasting developmental growth programs: skotomorphogenesis (etiolation) in the dark and photomorphogenesis (de-etiolation) under light. Etiolated seedlings grown in the dark display long hypocotyl, closed and yellowish cotyledons. On the other hand, when grown under light, plants develop de-etiolated morphology including short hypocotyl, open, expanded and green cotyledons (Gommers and Monte 2018). The transition from skoto- to photo-morphogenesis is regulated by a diverse group of photoreceptors that perceives and responds to different wavelengths of ambient light (Galvão and Fankhauser 2015). Among those photoreceptors, phytochromes function as pivotal sensors for red/far-red region of the light spectrum by allosterically altering the conformation from an inactive (Pr) to the active (Pfr) form (Bae and Choi 2008). The active Pfr form translocates into the nucleus (Klose *et al.* 2015) and interacts with multiple proteins including a small family of basic helix-loop-helix (bHLH) transcription factors called PHYTOCHROME INTERACTING FACTORS (PIFs) (Castillon *et al.* 2007; Huq and Quail 2005; Leivar and Quail 2011). These interactions result in a transcriptional reprogramming to switch to photomorphogenic development (Quail 2007).

Previous genetic studies have shown that each of the four major PIFs (PIF1, PIF3, PIF4 and PIF5) function distinctly as well as redundantly in controlling early seedlings development (Castillon*, et al.* 2007; Leivar and Monte 2014; Leivar and Quail 2011). In an overlapping manner, PIF1, PIF3, PIF4 and PIF5 inhibit photomorphogenic development in darkness as *pifQ* mutant display constitutive photomorphogenic phenotypes in the dark (Leivar *et al.* 2008; Shin *et al.* 2009) (Paik *et al.* 2017). Individually, PIF3 functions as a negative regulator of seedling de-etiolation and chlorophyll biosynthesis (Kim *et al.* 2003; Ogawa *et al.* 2003; Stephenson *et al.* 2009). It also positively regulates anthocyanin biosynthesis in response to light (Shin *et al.* 2007). PIF1 negatively regulates light-induced seed germination and chlorophyll biosynthesis (Huq *et al.* 2004; Oh *et al.* 2004). PIF4 acts as a negative regulator of red and far-red light signaling at the seedling stage (Huq and Quail 2002; Lorrain *et al.* 2009). Along with PIF5, PIF4 also regulates shade avoidance responses (Hersch *et al.* 2014; Lorrain *et al.* 2008), leaf senescence (Sakuraba *et al.* 2014; Song *et al.* 2014; Zhang *et al.* 2015), anthocyanin biosynthesis (Liu *et al.* 2015), and diurnal growth patterns (Kunihiro *et al.* 2011; Nozue *et al.* 2007). Recently, a number of studies have shown that PIF4 plays a critical role in the multiple signal integration, including light, temperature and hormonal signaling to regulate plant growth and development (Franklin *et al.* 2011; Gangappa *et al.* 2017; Kumar *et al.* 2012; Paik*, et al.* 2017). Apart from overlapping functions with PIF4, PIF5 acts as a negative regulator of red light signaling and as a positive regulator of ethylene biosynthesis (Khanna *et al.* 2007). Thus, PIF4 and PIF5 function as an integrator of multiple signaling pathways to optimize the growth and development.

In addition to PIFs, another group of negative regulators of photomorphogenesis is called CONSTITUTIVE PHOTOMORPHOGENIC/DEETIOLATED/FUSCA (COP/DET/FUS) (Lau and Deng 2012; Xu *et al.* 2015). These proteins have been shown to function as negative regulators of photomorphogenic development as their loss-of-function mutants develop as light-grown plants even under darkness (Deng *et al.* 1992; Lau and Deng 2012). COP1-SPA complex acts not only independently but also synergistically with PIFs to destabilize HY5, HFR1 and possibly other positive regulators to prevent photomorphogenesis in darkness (Hoecker 2017; Li *et al.* 2015; Xu *et al.* 2017). To promote photomorphogenesis, phytochromes repress these negative regulators through several distinct mechanisms. Phytochromes inhibit COP1-SPA complex by rapidly inducing their reorganization (Lu *et al.* 2015; Sheerin *et al.* 2015; Xu*, et al.* 2015) and/or nuclear exclusion under prolonged light (Subramanian *et al.* 2004). On the other hand, phytochromes inhibit PIF functions by inducing their degradation through the ubiquitin-proteasome system (UPS)-mediated pathway and also through sequestration of PIFs, eventually preventing them from binding to their target gene

promoters (Park *et al.* 2018; Park *et al.* 2012; Pham *et al.* 2018). Thus, phytochromes induce rapid destabilization of the negative regulators (e.g., PIFs) and stabilization of the positive geulators (e.g., HY5, HFR1 and others) to promote photomorphogenesis.

For post-translational regulation of PIF abundance, a number of kinases and E3 Ubiquitin ligases have recently been described (Pham*, et al.* 2018). Among the E3 ligases, $CUL3^{LRB}$ induces PIF3 degradation in response to light, where the LIGHT-RESPONSE Bric-a-Brack/Tramtrack/Broad (LRB1, LRB2, and LRB3) acts as substrate recognition components (Ni *et al.* 2014). Furthermore, a recent study showed PIF3 is ubiquitinated and degraded by the $SCF^{EBF1/EBF2}$ Ubiquitin ligase (Dong *et al.* 2017), where EIN3-BINDING F BOX PROTEIN 1 (EBF1) and EBF2 act as substrate recognition components. CUL3-based E3 ligase is also involved in the ubiquitination and degradation of PIF4 using the BLADE ON PETIOLE (BOP1/2) as the substrate recognition components (Zhang *et al.* 2017). CUL4-based E3 Ubiquitin ligase with the substrate recognition components, COP1-SPA promotes the light-induced ubiquitination and degradation of PIF1 in response to light (Zhu *et al.* 2015). In addition, $CUL1^{CTG10}$ promotes the degradation of PIF1 to regulate seed germination (Majee *et al.* 2018). In this case, COLD TEMPERATURE GERMINATING10 (CTG10), an F-box protein acts as a substrate recognition component. However, an E3 Ubiquitin ligase for PIF5 has not been described yet. Here, we show that $CUL4^{COP1\text{-}SPA}$ E3 ubiquitin ligase promotes the light-induced ubiquitination and subsequent degradation of PIF5 in red light. Light promotes *in vivo* interaction between COP1-SPA and PIF5 through the active phyB binding domain (APB). Strikingly, our results also suggest that COP1-SPA complex plays an essential role in controlling the photoperiodic hypocotyl growth, partly through post-translational regulation of the PIF5 abundance. Taken together, we propose a model where dynamic regulation of PIF5 by COP1-SPA complex optimizes not only seedling de-etiolation, but also diurnal regulation of plant growth and development in response to light.

RESULTS

CUL4 $^{COP1\text{-}SPA}$ complex promotes the degradation of PIF5 in response to red light

PIF5 is relatively stable in the dark; however, light signal induces rapid phosphorylation and subsequent degradation of PIF5 in a phytochrome-dependent manner (Shen *et al.* 2007). phyA, phyB and phyD regulate the light-induced degradation of PIF5 in a redundant manner, as *phyAB* and *phyABD* mutants show significantly delayed PIF5 degradation under red light. Moreover, the light-induced degradation of PIF5 is reduced by treatment with the proteasome inhibitor, suggesting that PIF5 is degraded through the 26S proteasome pathway (Shen*, et al.* 2007). However, the E3 Ubiquitin ligase necessary for PIF5 degradation is still unknown. Because there are hundreds of possible combinations of E3 Ubiquitin ligases in plants (Vierstra 2009), we focused on three CULLIN mutants (*cul1-6*, *cul3ab* and *cul4cs*) to narrow down the possible substrate adaptor components required for PIF5 degradation. We performed immunoblots to detect PIF5 abundance in *cul4cs* (Chen *et al.* 2010; Zhang *et al.* 2008)*, cul1-6* (Moon *et al.* 2007), and *cul3ab* (Thomann *et al.* 2005) mutant seedlings, grown in the dark and dark-grown seedlings exposed to red light. It was observed that the rate of degradation of PIF5 is slower only in the *cul4cs* background but not in the *cul1-6* or *cul3ab* mutant backgrounds (Figure S1). These data suggest that CUL4-based E3 Ubiquitin ligase promotes the degradation of PIF5 during early light responses.

Recently, we have shown that CUL4$^{COP1\text{-}SPA}$ complex promotes the ubiquitination and degradation of PIF1 in response to light (Zhu*, et al.* 2015). To examine whether COP1-SPA complex induces the degradation of PIF5, we performed immunoblots to test the degradation rate of PIF5 in wild-type (Col-0), *cop1-4* and *spaQ* mutants in response to red light. As reported in our earlier studies, PIF5 level is significantly lower in both *cop1-4* and *spaQ* mutants in the dark (Xu*, et al.* 2017). However, the light-induced degradation is significantly reduced in both *cop1-4* and *spaQ* mutants (Figure 1A, B, C; Figure S2). COP1 appears to play a more prominent role in regulating PIF5 compared to SPA proteins, as PIF5 is more stable in *cop1-4* than *spaQ* background. We also tested PIF5 level under far-red light and shade mimicking (low R/FR ratio) conditions. As consistent with previous studies, PIF5 is stable under far-red and the low R/FR ratio conditions (Figure S3) (Lorrain*, et al.* 2008). To examine if the red light-induced reduction in PIF5 level is due to a reduction of *PIF5* transcript level, we performed qRT-PCR for *PIF5* transcript. The results show that *PIF5* mRNA level is significantly higher in both *cop1-4* and *spaQ* mutants, compared to wild -type both in the dark and red light, possibly due to the constitutively photomorphogenic phenotypes of these mutants (Figure S4A). However, *PIF5* mRNA level is comparable between dark and 30 min of red light treated

samples of wild-type and *cop1-4* mutant, suggesting that light does not regulate *PIF5* expression under these conditions in these backgrounds (Figure S4A). These data clearly illustrate that the light signal triggers the degradation of PIF5 protein through the $CUL4^{COP1\text{-}SPA}$ complex.

We also tested PIF5 protein level in *cop1-4* and *spaQ* mutants under prolonged red light conditions. Since, *PIF5* expression is regulated by circadian clock, we crossed *35S:PIF5-Myc* into *cop1-4* and *spaQ* mutants (PIF5-Myc/*cop1-4* and PIF5-Myc/*spaQ*) and PIF5-Myc degradation patterns is examined under prolonged light conditions (Figure S5). The level of PIF5 protein in wild-type is reduced ~50% within 10 min under Rc (2 $\mu molm^{-2}$ s^{-1}) and reached the trough level after 3hrs of continuous red light treatment. Interestingly, starting at 6 hrs, PIF5 level increases to peak at 24 hrs under continuous red light. Re-accumulation of PIF5 at subjective dawn under continuous light suggests that PIF5 protein level might be regulated by the circadian clock or diurnally (Figure S5A). To test whether PIF5 re-accumulates in the *cop1-4* and *spaQ* backgrounds, we performed immunoblots for PIF5-Myc in these backgrounds (Figure S5B, C). The results show that the light-induced degradation of PIF5 is reduced in these backgrounds compared to wild-type. Moreover, the re-accumulation of PIF5 under continuous red light at presumptive dawn is also defective in these mutants. These data suggest that COP1 and SPA proteins are involved in regulating PIF5 level under both short-term and prolonged light conditions.

PIF5 target gene expression is affected in *cop1-4* and *spaQ*

Since PIF5 is a transcription factor that regulates the expression of genes involved in the light response pathways (Kunihiro*, et al.* 2011; Liu *et al.* 2013; Sakuraba*, et al.* 2014), we tested whether PIF5 target gene expression is affected in *cop1-4* and *spaQ* mutants using qRT-PCR assays for three PIF5-induced genes (*AT5G02580*, *ATHB2* and *ARF8*) and one PIF5-repressed gene (*RGF9*) (Zhang *et al.* 2013). The wild type dark level of expression for each gene was set at "1" and then the relative fold-change in expression of these genes in *cop1-4* and *spaQ* backgrounds under darkness and dark-grown seedlings exposed to 30 min of R light were calculated. Consistent with the PIF5 protein level in these mutants, the qRT-PCR data show that the fold-change in PIF5 target gene expression in response to red light is reduced in *cop1-4* and *spaQ* compared to wild-type (Fig. S4B). This is consistent with the reduced PIF5 degradation in *cop1-4* and *spaQ* compared to wild type under red light.

PIF5 is ubiquitinated via 26S proteasome pathway in response to light

Light–induced degradation of PIFs is mediated through the UPS pathway and involves rapid phosphorylation and ubiquitination of PIFs (Ni*, et al.* 2014; Shen *et al.* 2008; Zhu*, et al.* 2015). To determine whether PIF5 is ubiquitinated in response to light, we immunoprecipitated PIF5-Myc from dark-grown (Dk) seedlings and dark-grown seedlings treated with continuous red light for 30 min (R) and then performed immunoblots using anti-Myc and anti-Ubi antibodies (Figure 2A, right panel). The immunoblots show that PIF5 is strongly ubiquitinated in response to the light.

We also examined whether the light-induced ubiquitination of PIF5 is affected in *cop1-4* and *spaQ* mutants in the dark and red light. Four-day-old dark-grown seedlings overexpressing *PIF5* in these backgrounds were either kept in the dark or exposed to red light for the indicated period of time. One of the samples was pretreated with a protease inhibitor (40 μM Bortezomib) before exposing to red light for the indicated time. The samples with Bortezomib treatment show strong ubiquitination pattern, which suggests that the protease inhibitor efficiently inhibited the degradation of PIF5 under red light, thereby, increased the ubiquitinated PIF5 level (Figure 2A). Strikingly, the level of PIF5 ubiquitination is reduced in the *cop1-4* and *spaQ* backgrounds compared to wild-type (Figure 2B, C). The phosphorylation of PIF5 is not observable under these conditions for this tagged version. These data suggest that COP1 and SPA proteins promote the ubiquitination of PIF5 to induce the rapid degradation in response to red light. Previous studies showed that PIFs are not directly ubiquitinated by COP1 *in vitro* (Jang *et al.* 2010; Xu *et al.* 2014). However, PIF1 is ubiquitinated by the $CUL4^{COP1\text{-}SPA}$ complex in response to light *in vivo* (Zhu*, et al.* 2015). Thus, it is possible that the light-induced degradation of PIF5 also requires $CUL4^{COP1\text{-}SPA}$ complex similar to PIF1.

PIF5 interacts with COP1 and SPA1 in yeast and *in vivo*

To examine if PIF5 can interact with COP1 and SPA1, we performed yeast two-hybrid (Y2H) assays using full-length PIF5 and COP1-SPA1 proteins. We also created a mutation in the APB motif of PIF5 (PIF5 G37A) and used it in Y2H assays. This residue was shown to be necessary for the physical interaction between PIF5 and phyB (Khanna *et al.* 2004). Results show that the wild-type PIF5 can interact with both COP1 and SPA1 proteins (Figure 3A). However, the G37A mutation in PIF5 reduced the interaction with both COP1 and SPA1. These results suggest that the APB domain of PIF5 is indeed necessary for the interaction with COP1 and SPA1.

In vitro interaction between PIF5 and COP1 has been described earlier (Jang*, et al.* 2010). To test *in planta* interaction, we performed transient luciferase complementation imaging assay (LCI) in tobacco (*Nicotiana benthamiana*) leaves*.* Similar to Y2H assays, it was observed that the PIF5 but not the PIF5 (G37A) interact with both COP1 and SPA1 (Figure 3B). These data suggest that PIF5 interacts with COP1 and SPA1 *in planta*.

To promote ubiquitination and degradation of PIF5 in response to light, COP1-SPA1 interaction with PIF5 is expected to be induced by light. To test this possibility, we performed *in vivo* co-immunoprecipitation (Co-IP) assays using seedlings grown in the dark for four days and four-day-old dark-grown seedlings exposed to red light (3000 $\mu molm^{-2}$). The results show that PIF5 can interact with both COP1 and SPA1, and that the strength of interaction is significantly enhanced by red light treatment (Figure 3C, 3D). Taken together these results clearly indicate that PIF5 does interact with COP1 and SPA1 and these interactions are regulated by light.

PIF5 partially suppresses the *cop1* and *spaQ* phenotypes

To examine the significance of PIF5 degradation by the COP1-SPA complex, we tested the phenotypes of the PIF5-Myc/*cop1-4* and PIF5-Myc*/spaQ* compared to those of *cop1-4* and *spaQ* phenotypes under both dark and red light conditions. Since PIF5 functions as a negative regulator of photomorphogenesis in the dark, increasing the level of PIF5 in the *cop1* and *spaQ* is expected to suppress photomorphogenesis. In the dark, overexpression of *PIF5* mimics triple responses including short hypocotyl and exaggerated apical hooks, due to the induction of ethylene biosynthesis in seedlings (Khanna*, et al.* 2007). Strikingly, the hypocotyl length of PIF5-Myc/*cop1-4* seedlings growing in the dark is significantly longer than *cop1-4* (Figure 4A, C). The hypocotyl length of PIF5-Myc/*spaQ* is slightly longer compared to *spaQ* in the dark (Fig. 4A, C). In addition, overexpression of *PIF5* in the *cop1-4* and *spaQ* partially repressed the cotyledon opening phenotype of both *cop1-4* and *spaQ* in the dark (Figure 4E). Similarly, overexpression of *PIF5* in *cop1-4* mutant results in slightly longer hypocotyl than *cop1-4* mutant under continuous red light (Figure 4B, D). However, the hypocotyl length of PIF5-Myc/*spaQ* is similar to that of *spaQ* under continuous red light. By contrast, the cotyledon opening angle of both *cop1-4* and *spaQ* is partly suppressed in PIF5-Myc/*cop1-4* and PIF5-Myc/*spaQ* seedlings (Figure 4F).

Earlier it was shown that the PIF5 also function as a negative regulator of anthocyanin accumulation (Liu*, et al.* 2015); thus, light-induced degradation of PIF5 is expected to relieve this inhibition to promote anthocyanin biosynthesis. In the agreement with the previous study, during the dark to light transition, *PIF5* overexpressed seedlings display very low level of anthocyanin. However, *cop1-4* and *spaQ* mutants display a high level of anthocyanin. Interestingly, *PIF5* overexpression in *cop1-4* and *spaQ* mutants exhibits much less anthocyanin content compared to *cop1-4* and *spaQ* both in the dark and light-exposed seedlings (Figure 4G). Thus, PIF5 partially suppresses the hypocotyl length, cotyledon opening angle, and anthocyanin level phenotypes of the *cop1-4* and *spaQ* mutants.

Photoperiodic control of hypocotyl growth by COP1-SPA complex via PIF5

The diurnal regulation of hypocotyl elongation by PIFs have been well documented (Ezer *et al.* 2017; Nozue*, et al.* 2007; Nusinow *et al.* 2011; Soy *et al.* 2016). However, it is still unknown whether COP1 and SPA proteins regulate diurnal growth patterns in a photoperiodic manner. To test this, we first measured the hypocotyl elongation phenotype under short day (SD) conditions. Overexpression of *PIF5-Myc* in seedlings displayed much longer hypocotyl, while *cop1-4* and *spaQ* mutants showed much shorter hypocotyl compared to wild-type (Figure 5A, B). However, when PIF5-Myc was introduced into the *cop1-4* and *spaQ* backgrounds, the hypocotyl lengths are partly rescued compared to the *cop1-4* and *spaQ* mutant backgrounds (Figure 5A, B, Figure S6). These data are qualitatively very similar to those under continuous red light conditions. To measure the growth defect in these mutants, we measured growth rate using an infrared imaging system (Nozue*, et al.* 2007). The hypocotyl growth rate was measured starting from day 2, when the hypocotyl lengths are visible. In Col-0 and PIF5-Myc seedlings, the growth rate reaches the peak during night-time (ZT18-ZT24). In *pif5* and *pif45* mutants, the growth rate is reduced as previously shown (Nozue*, et al.* 2007). Strikingly, the growth rate is impaired in the *cop1-4* and *spaQ* mutants during these periods *(*Figure 5C). However, overexpression of *PIF5* in these mutant backgrounds partly rescues the growth rate under these conditions, suggesting that PIF5 acts downstream of these factors to regulate diurnal growth rate.

To correlate growth rate with PIF5 abundance, we measured PIF5-Myc protein and mRNA levels in the wild-type, *cop1-4* and *spaQ* mutants under the same conditions (Figure 6A-D). At dawn, light treatment triggers the degradation of PIF5, so that the level of PIF5 dropped at a minimum level at ZT4-8. PIF5 started to re-accumulate at the beginning of the dark period, reaching the maximum

level after 16-20h of dark (ZT18-ZT24) (Figure 6A-C). The maximum level of PIF5 protein at the end of the dark period correlates well with the growth rate in PIF5-Myc seedlings supporting the role for PIF5 in diurnal growth regulation (Figure 5C). However, in *cop1-4* and *spaQ* mutants, the level of PIF5 protein was significantly reduced under both prolonged white light and dark period. Moreover, the PIF5 protein level failed to reach the peak at ZT18-ZT24 (Figure 6A-C). Therefore, PIF5 oscillation pattern is dramatically impaired in the *cop1-4* and *spaQ* mutant backgrounds compared to wild-type. Contrary to protein level, qRT-PCR data show that there is little or no significant difference in *PIF5* mRNA level in wild-type, *cop1-4,* and *spaQ* (Figure 6D). These data suggest that the defect in PIF5 and possibly other PIF levels in the *cop1-4* and *spaQ* backgrounds might correlate with the growth defects in *cop1-4* and *spaQ* under diurnal conditions.

PIF5 has been shown to regulate growth-related *ATHB2* and *IAA29* gene expression during diurnal growth (Kunihiro*, et al.* 2011). To examine if the expression of these genes correlate with the growth defects in *cop1-4* and *spaQ* mutants, we performed qRT-PCR for these genes in the wild-type, *cop1-4* and *spaQ* backgrounds grown under SD conditions. Surprisingly, the expression level of these two genes is significantly impaired during ZT20-24 in PIF5-Myc/*cop1-4* and PIF5Myc/*spaQ* mutants compared to PIF5-Myc seedlings (Figure 6E-F). Taken together, these data illustrate that COP1-SPA complex is involved in regulating diurnal growth pattern via controlling PIF5 level.

DISCUSSION

$CUL4^{COP1\text{-}SPA}$ complex promotes the light–induced degradation of PIF5

The genetic, biochemical and photobiological data presented here strongly suggest that $CUL4^{COP1\text{-}SPA}$ complex functions as an E3 Ubiquitin ligase for the light-induced degradation of PIF5. First, the light-induced degradation of PIF5 is reduced in the *cul4cs, cop1-4* and *spaQ* mutants compared to wild-type. Second, both COP1 and SPA1 interact with PIF5 in a light stimulated manner. Third, the light-induced ubiquitination of PIF5 is defective in *cop1-4* and *spaQ* backgrounds. Fourth, overexpression of *PIF5* suppresses the photomorphogenic phenotypes of *cop1-4* and *spaQ* mutants. Taken together, these data suggest that COP1 and SPA proteins act as substrate recognition factors to recruit PIF5 in the $CUL4^{COP1\text{-}SPA}$ E3 ubiquitin ligase for the light-induced ubiquitination and subsequent degradation.

Different E3 ligase for the degradation of different PIFs

Three main classes of CULLIN (CUL) RING UBIQUITIN LIGASEs (CRLs) have been shown to be involved in the light-induced degradation of PIFs (Pham*, et al.* 2018). $CUL1^{EBF1/2}$ and $CUL3^{LRB}$ have been shown to regulate PIF3 abundance (Ni*, et al.* 2014), while $CUL3^{BOP1/2}$ promotes the degradation of PIF4 (Zhang*, et al.* 2017). $CUL1^{CTG10}$ and $CUL4^{COP1\text{-}SPA}$ complexes induce the degradation of PIF1 in response to light to promote seed germination (Majee*, et al.* 2018; Zhu*, et al.* 2015). Our study provides evidence that $CUL4^{COP1\text{-}SPA}$ Ubiquitin ligase is also involved in the ubiquitination and degradation of PIF5. The light-induced degradation of PIF5 does not appear to be defective in the *cul1-6* and *cul3ab* mutants under these conditions. This does not preclude their involvement in regulating PIF5 under other conditions. In fact, PIF5 is still degraded in the *cop1-4* and *spaQ* mutants under prolonged light conditions, suggesting that other E3 ubiquitin ligases might also be involved at those conditions.

A trend appears when we compare the rate of light-induced degradation of different PIFs and the involvement of different E3 ubiquitin ligases. Among all PIFs, the rate of degradation of PIF1 and PIF5 is the fastest with a half-life of <5 min under red light (Shen*, et al.* 2008; Shen*, et al.* 2007). However, the light-induced degradation of PIF3 and PIF4 is relatively slower than that of PIF1 and PIF5 (Al-Sady *et al.* 2006; Lorrain*, et al.* 2008). Thus, $CUL4^{COP1\text{-}SPA}$ complex might be involved in early and rapid light-induced degradation of PIF1 and PIF5, whereas other E3 Ubiquitin ligases are involved in the degradation of other PIFs, either at a later time and/or under higher irradiation conditions. The facts that both PIF1 and PIF5 bind to COP1 and SPA1 in the dark, and their physical interaction is enhanced by light, suggest that the $CUL4^{COP1\text{-}SPA}$ E3 Ubiquitin ligase is poised to initiate the ubiquitination process (Xu*, et al.* 2014; Zhu*, et al.* 2015). This is also the case for PIF3-EBF1/2 interaction; however, the SCF formation requires the light-induced phosphorylation, suggesting that $SCF^{EBF1/2}$ is not functional in the dark (Dong*, et al.* 2017). These differences might partly explain the faster rate of degradation of PIF1 and PIF5 compared to the other PIFs under these conditions. Regardless of these details, the division of labor for PIF degradation among different E3 Ubiquitin ligases ensures temporal distribution of ubiquitin ligase machineries to optimize the inhibition of skotomorphogenesis and promotion of photomorphogenesis.

PIF5 is not only degraded under light but also in darkness

PIF5 level is much lower in the *cop1-4* and *spaQ* mutants compared to wild-type in the dark, suggesting that COP1-SPA might play a role in stabilizing PIF5 in the dark. Other PIFs including PIF1 and PIF3 are also unstable in the *cop1-4* and *spa123* backgrounds in the dark (Bauer *et al.* 2004; Leivar*, et al.* 2008; Ni*, et al.* 2014). A recent study showed that COP1-SPA complex stabilizes PIF3 by inhibiting the phosphorylation of PIF3 by BIN2 (Ling *et al.* 2017). We have also shown that the increased abundance of HFR1 in the *cop1-4* mutant induces degradation of PIF1 and PIF5 in the dark in a heterodimerization-dependent manner (Xu*, et al.* 2017). Collectively, these data suggest that PIF5 is degraded either directly or indirectly in *cop1-4* and *spaQ* background in the dark through the 26S proteasome pathway.

COP1-SPA complex regulates hypocotyl growth under diurnal conditions

PIF5 along with PIF4 functions as an integrator of light and circadian clock, where circadian clock regulates the expression of *PIF5* and *PIF4*, while light signal regulates the PIF5 and PIF4 protein abundance (Nozue*, et al.* 2007). Circadian clock regulates the diurnal plant growth including hypocotyl elongation, which is more pronounced under short-day (SD) conditions. During early night, the expression of *PIF5* and *PIF4* is inhibited by the ELF3-ELF4-LUX evening complex (Nusinow*, et al.* 2011). However, closer to dawn, the clock-mediated repression of *PIF5* and *PIF4* expression is suppressed. The increased expression of *PIF5* and *PIF4* induces hypocotyl growth during the end of the night. In consistent with these data, diurnal growth defect in *pif4pif5* mutant has been well documented (Ezer*, et al.* 2017; Nozue*, et al.* 2007; Nusinow*, et al.* 2011). PIF3 is also involved in diurnal growth control; however, the mechanism is different. In this case, TOC1 interacts with PIF3 during early evening and inhibits PIF3 function. At dawn, *TOC1* expression is reduced, which relieves this inhibition resulting in the promotion of hypocotyl growth by PIF3 (Soy*, et al.* 2016). In fact, a wave of PSEUDO-RESPONSE REGULATORS (PRR9/7/5) also inhibits PIF activity to repress growth during the day time (Martín *et al.* 2018). Although, PIFs are at the center stage for diurnal regulation of growth, a growth defect for *cop1* and *spaQ* mutants under diurnal conditions have not been shown yet. Our data show that *cop1* and *spaQ* mutants are not only defective under continuous dark and light conditions, but also under diurnal conditions. Strikingly, this defect is also mediated through regulating PIF5 level post-translationally under these conditions. As previously shown, PIF5 level increases post-translationally toward the end of the dark period under diurnal conditions. However, this

increase is defective in the *cop1-4* and *spaQ* mutants, suggesting COP1-SPA complex stabilizes PIF5 under these conditions. Moreover, overexpression of *PIF5* in the *cop1-4* and *spaQ* backgrounds partially rescues the diurnal growth defect of *cop1-4* and *spaQ* mutants compared to wild-type. Overall, these data suggest that COP1-SPA complex is involved in regulating diurnal growth pattern through dynamic regulation of PIF5 and possibly other PIF levels.

MATERIALS AND METHODS

Plant growth conditions

Seeds were surface sterilized with 10% commercial bleach and 0.3% SDS for 5 min, washed 5 times with water and then plated on Murashige–Skoog (MS) growth medium containing 0.9% agar without sucrose. Plates were kept in the dark for 3 days at 4°C to stratify. Plates were then exposed to white light for 3hrs at room temperature before being placed in the dark or red light for 4 days. Specific light conditions for each of the assays are described in figure legends.

For high R/FR and low R/FR conditions, seedlings were grown under short-day (8hrs of high R/FR light/16hrs dark) for 5 days. At dawn on day 6^{th}, seedlings were either kept in high R/FR condition (R/FR =4.51) or transferred to low R/FR condition. Low R/FR condition was provided by supplementary FR light (λ=735 nm) (R/FR = 0.1) (Lorrain*, et al.* 2008).

Measurement of hypocotyl lengths and anthocyanin content

For the measurement of hypocotyl length and cotyledon opening angle under dark and red light ($2\mu molm^{-2}s^{-1}$), images of 90 seedlings (30 seedlings for each experiment with 3 independent biological replicates) were taken and then measured using the publicly available ImageJ software (http://rsb.info.nih.gov/ij/). For the measurement of anthocyanin content, 50 seeds were plated on MS medium containing 2% sucrose and kept in the dark for 3 days at 4°C. Seeds were exposed to 3hrs of white light to induce germination and then kept in the dark for 2 days. One set of seedlings was kept in the dark and another was transferred to continuous white light and grown for another 2 days. Anthocyanin content was measured following the protocol described previously (Neff and Chory 1998; Schmidt and Mohr 1981). Anthocyanin content was calculated by [Abs_{530} - (0.25 x Abs_{657})]. Data represents the anthocyanin content per gram fresh weight.

Construction of vectors and generation of transgenic plants

Preparation of PIF5-Myc transgenic line (35S:PIF5-Myc/Col-0) was described previously (Sakuraba*, et al.* 2014). PIF5-Myc transgenic line was crossed with *cop1-4* and *spaQ* mutants. PIF5-Myc/*cop1-4* and PIF5-Myc*/spaQ* homozygous lines were selected from F3 population. Primer used for *cop1-4* mutant sequencing and *spaQ* genotyping are listed in Table S1.

To prepare double transgenic line *PIF5-HA/TAP-SPA1,* full length PIF5 CDS without a stop codon was amplified from a cDNA library using primers listed in Table S1, and directionally cloned into pENTR vector (Invitrogen Inc., Carlsbad, CA). Coding sequence was subcloned into *pGWB14* binary vector (Karimi *et al.* 2005) and then transformed into *Agrobacterium tumefaciens* strain GV3101. This construct was then transformed into p*35S:TAP-SPA1* transgenic plants using *Agrobacterium*-mediated transformation as previously described (Clough and Bent 1998) and double homozygous lines were selected in T3 generation.

Preparation of constructs for LCI assay: pCAMBIA split luciferase vectors were kindly provided by Dr. Jian-Min Zhou (Chen *et al.* 2008). p*35S: nLUC* vector was digested with EcoRI/HindIII and ligated into *pZP121* vector to create *pZP121: nLUC* vector. For COP1-PIF5 interaction, full length *COP1* CDS was cloned into KpnI/SalI sites of the *PZP121-nLUC* and full length *PIF5/PIF5-G37A* CDS was cloned into KpnI/SalI sites of *pCAMBIA1300-cLUC* vector.

For PIF5-SPA1 interaction, SPA1 coding sequence was cloned into EcoRI/ XhoI sites of *pCAMBIA1300-cLUC*. *PIF5* and *PIF5-G37A* coding sequencing were cloned into KpnI/SalI sites of the *pCAMBIA1300-nLUC* vectors. The sequences of *pCAMBIA1300-nLUC* and *pCAMBIA1300-cLUC* vectors are available on TAIR website. All the constructs were confirmed by sequencing. The *PIF5-G37A* mutation was introduced using a site-directed mutagenesis kit (Stratagene, La Jolla, CA). Primers used for vector constructions and also site-directed mutagenesis are listed in Table S1.

Luciferase complementation imaging (LCI)

Luciferase complementation imaging (LCI) for testing the interaction of PIF5 with COP1 and SPA1 in *N. benthamiana* was performed as previously described with few modifications (Chen*, et al.* 2008). *A. tumefaciens* strain GV3101 was used for the injection of different constructs into *N. benthamiana* leaves. 1mM Luciferin was sprayed into the *N. benthamiana* leaves and kept in the

dark for 5 min. Low-light cooled CCD camera (NightOWL IILB 983 NC 100, BERTHOLD) was used to capture luciferase luminesence. An exposure time of 2 min with 2x2 binning was used. Relative luciferase was equivalent to luminescence intensity / 0.2mm^2 leaf area.

Yeast two-hybrid and quantitative β-galatosidase assays

Full-length PIF5 and PIF5-G37A coding sequences were amplified by PCR using the primers listed in Table S1. PIF5 and PIF5-G37A CDS were then cloned into EcoRI/ SalI sites of the LexA vector (pEG202) containing the DNA binding domain. COP1 and SPA1 were cloned into EcoRI/XhoI sites of the activation domain vector (pB42AD). These plasmids were transformed into yeast strain EGY48 and selected on dropout medium without Uracine, Leucine and Tryptophan. The quantitative β-galactosidase assay was performed according to the manufacturer's instructions (Matchmaker Two-Hybrid System, Clonetech Laboratories Inc.,).

Protein extraction and immunoblotting

To analyze PIF5 abundance in wild-type and different mutant seedlings, total protein was extracted in denaturing buffer as described earlier (Zhu*, et al.* 2015). Total protein was separated on 8% SDS-PAGE gel and transferred to PVDF membrane. Membranes were blotted with anti-Myc (dilution 1/1000, Catalog number OP10-200UG, EMD Millipore, Billerica, MA) or anti-PIF5 (AS122112, Agrisera, Vannas, Sweden) or anti-RPT5 (dilution 1/3000, Catalog number: BML-PW8245-0100, Enzo Life Sciences, Farmingdale, NY) to detect PIF5-Myc, endogenous PIF5 and RPT5, respectively. In order to provide the true-dark condition, seeds were treated with far-red light for 5 min (6.5 μmolm^{-2}s^{-1}) after 21hrs in the dark and put them back to the dark for an additional 3 days.

For ubiquitination assays 4-day-old dark-grown seedlings were either kept in the dark or exposed to the constant red (Rc; 2 μmolm^{-2}s^{-1}) for 15 and 30 min. One batch of samples from each genotype tested were pretreated with 40 μM Bortezomib (B-1408, LC Laboratories, Woburn, MA) for 4 hrs before being exposed to 30 min Rc. Total protein from 50 seedlings were extracted in 50μl of urea extraction buffer (48% urea (w/v), 0.1M phosphate buffer pH 6.8, 10mM Tris-Cl pH 6.8, 1 mM PMSF and 1× protease inhibitor cocktail). Samples were centrifuged at 16,000g for 10 min and the supernatant was collected. The supernatant was boiled for 10 min at 65°C and were resolved on 6.5 % SDS-PAGE gels. Proteins were transferred to PVDF membranes and blotted with anti-Myc and anti-RPT5.

***In vivo* Co-immunoprecipitation assays**

For detecting the ubiquitination pattern of PIF5-Myc *in vivo*, four-day-old dark-grown seedlings were kept in the dark or treated with 2 $\mu molm^{-2}\ s^{-1}$ constant red light (Rc) for 30 min. Total protein was extracted in native buffer as described previously (Zhu*, et al.* 2015) and PIF5-Myc was immunoprecipitated using anti-Myc antibody (1μg, 71D10, Cell Signaling Technologies, Danvers, MA). Samples were resolved on 6.5% SDS-PAGE, transferred to PVDF membrane and blotted with anti-Myc (1/1000 dilution, EMD Milliore, Billerica, MA) and anti-Ubi antibodies (1/1000 dilution, sc-8017, Santa Cruz Biotechnology, Santa Cruz, CA).

To perform *in vivo* Co-IP assays 5-day-old dark-grown seedlings were pretreated with 40μM bortezomib for 4hrs and then treated with either dark or red light (3000 $\mu molm^{-2}$). Total protein was extracted in native buffer and immunoprecipitation was performed as described earlier (Zhu*, et al.* 2015). For COP1-PIF5 interaction assays, total protein was isolated from *PIF5-Myc* seedlings. PIF5-Myc was immunoprecipitated using anti-Myc (EMD Milliore, Massachusetts) antibody and interacting partner COP1 was detected using anti-COP1 antibody. For SPA1-PIF5 interaction, total protein was isolated from *TAP-SPA1/PIF5-HA* double transgenic lines. PIF5-HA was immunoprecipitated using anti-HA (1μg, catalog number ab9110, Abcam, Cambridge, MA) and interacting partner TAP-SPA1 was detected using anti-Myc antibodies (EMD Milliore, Billerica, MA).

RNA isolation and Quantitative RT-PCR assay

Total RNA was extracted from 4 day-old dark-grown seedlings or dark-grown seedlings exposed to 30 min of Rc (2 $\mu molm^{-2}\ s^{-1}$) using the Sigma spectrum total RNA kit (Sigma-Aldrich, St. Louis, MO). One μg of total RNA was treated with DNase 1 (NEB, Ipswich, MA) and reversed transcribed using M-MLV reverse transcriptase (ThermoFisher Scientific, Waltham, MA). qRT-PCR was performed using Power SYBR® green (Applied Biosystems, Foster City, CA) and gene specific primers, as listed in Table S1. *PP2A* was used as the control for normalization the expression of other genes. The cycle threshold (Ct) values were used for calculation of the levels of expression of different genes relative to *PP2A.*

Hypocotyl and growth rate measurement under SD condition

The infrared images were taken using the Marlin camera (Allied Vision Technologies, Exton, PA) with the lenses fitted with IR long-pass cut filters (NT54-755, Edmund Optics). Seedling samples were back-lit with an IR LED back-light emitting at 880 nm (Advanced Illumination). Seeds were grown under SD and the pictures were acquired at the beginning of day 2. Image acquisition was archived using AVT SmartView software (Allied Vision Technologies, Exton, PA) every 1h for 3 days. Images were analyzed using ImageJ tool. The hypocotyl growth rates were measured using 4 seedlings for each genotype at each time point, and three replicates have been done. The mean and SD shown in the graph indicates the mean and SD for one replicate.

AUTHOR CONTRIBUTIONS

V.N.P., P.K.K. and E.H. contributed to the experimental design. V.N.P. and P.K.K. carried out the experiments, V.N.P., P.K.K. and E.H. interpreted the results. V.N.P. and P.K.K. prepared the manuscript. V.N.P., P.K.K. and E.H. edited the manuscript.

ACKNOWLEDGEMENTS

We thank members of the Huq laboratory for critical reading of the manuscript, Drs. Giltsu Choi for sharing *myc-PIF5* seeds, Xing Wang Deng for *cop1-4* seeds, Ute Hoecker for *spaQ* seeds, Woe Yeon Kim for COP1 antibody and Jian-Min Zhou for pCAMBIA split luciferase vectors. This work was supported by grants from National Institute of Health (NIH) (1R01 GM-114297), National Science Foundation (MCB-1543813) and United States Israel Bilateral Science Foundation (2015316) to E. H.

The Authors declare no conflicts of interest.

SHORT SUPPLEMENTAL INFORMATION LEGENDS

Figure S1: Red light induced degradation of PIF5 is slower in *cul4cs* and but not in *cul1-6* and *cul3ab* mutants.

Figure S2: Prolonged red light induces the degradation of PIF5 in both *cop1-4* and *spaQ* mutants with slower rate compared to wild-type

Figure S3: PIF5 level is stable under far-red (FR) light and shade mimicking (low R/FR) conditions.

Figure S4: COP1-SPA post-translationally regulates PIF5 abundance both in the dark and red light

Figure S5: Fluctuation of the PIF5-Myc level during the prolonged red light treatment

Figure S6: Overexpression of *PIF5-Myc* partially rescue hypocotyl elongation in *cop1-4* and *spaQ.*

Table S1: Primer sequences used in experiments described in manuscript.

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FIGURE LEGENDS

Figure 1. Red light induced PIF5 degradation is slower in *cop1-4* and *spaQ* mutants

(A-B) Immunoblots showing the degradation pattern of endogenous PIF5 in response to dark and constant Rc in *cop1-4* (A) and *spaQ* (B) mutants, compared to the wild-type seedlings. Four-day-old dark-grown seedlings were treated with either dark (Dk) or 2 $\mu molm^{-2}\ s^{-1}$ constant red light (Rc) for the indicated period of time (min). Total protein was extracted and resolved on 8% SDS-PAGE gel. Proteins were transferred to PVDF membrane and sequentially probed with anti-PIF5 and anti-RPT5.

(C) Line-graph shows the relative rate of degradation of endogenous PIF5 in response to constant Rc over the dark treatment. Band intensities of PIF5 and RPT5 from three replicates were measured using ImageJ tool. For each of the genotype, PIF5 level in the dark (Dk) was set to 1and the relative PIF5 level in response to constant Rc was calculated.

Figure 2. Red light-induced ubiquitination of PIF5 is reduced in *cop1-4* and *spaQ* mutants

(A) Immunoblots showing the ubiquitination pattern of PIF5-Myc in response to dark or red light treatment. Four-day-old dark-grown seedlings were treated with either dark (Dk) or 2 $\mu molm^{-2}\ s^{-1}$ constant red light (R) for 30 min. Total protein was extracted in native buffer and PIF5-Myc was immunoprecipitated using anti-Myc antibody. Samples were resolved on 6.5% SDS-PAGE and transferred to PVDF membrane. Membranes were blotted with anti-Myc (Left panel) and anti-Ub (Right panel) antibodies.

(B-C) Immunoblots showing the relative ubiquitination status of PIF5-Myc in response to dark and constant Rc in *cop1-4* (B) and *spaQ* (C) mutants, compared to the wild-type. Four-day-old dark-grown seedlings were treated with either dark (Dk) or 2 $\mu molm^{-2}\ s^{-1}$ constant red light (Rc) for the indicated period of time (min). One of the samples in each of the genotype was pretreated with 40 µM proteasome inhibitor (Bortezomib) for 3 hrs prior to Rc treatment. Total protein was extracted and samples were resolved on 6.5% SDS-PAGE and transferred to PVDF membrane. Membranes were sequentially blotted with anti-Myc and anti-RPT5 antibodies.

Figure 3. PIF5 interacts with COP1 and SPA1 through the APB domain

(A) Quantitative yeast two-hybrid assay showing interactions of COP1 and SPA1 with wild-type PIF5 and Active phyB binding domain (APB) mutagenized PIF5 (PIF5G37A). PIF5 and PIF5G37A were cloned into LexA vector containing the DNA binding domain. COP1 and SPA1 were cloned into a vector containing activation domain (AD). Empty AD vector was used to read the background level.

Liquid β-galactosidase assays were performed in triplicate and Miller Units (M.U; measure of β-galactosidase activity) was determined.

(B) Split luciferase imaging (LCI) assay (Lower panel) and quantitative assay (Upper panel) showing *in vivo* interactions of COP1 and SPA1 with PIF5 in *N. benthamiana* leaves. Full-length PIF5, PIF5-G37A, COP1 and SPA1 were fused to either cLUC or nLUC as indicated in the figure. Empty vectors were used as negative controls.

(C and D) Co-immunoprecipitation (Co-IP) assay showing *in vivo* interaction of PIF5-Myc with COP1 (C) and PIF5-HA with TAP-SPA1 (D). Five-day-old dark-grown seedlings were pretreated with 40μM bortezomib for 4hrs and then treated with either dark or red light (3000 $\mu molm^{-2}$). Total protein was extracted in native buffer. PIF5-Myc and PIF5-HA were immunoprecipitated with anti-Myc and anti-HA antibodies, respectively. Interacting partners COP1 and SPA1 were detected using anti-COP1 and anti-Myc antibodies, respectively.

Figure 4. COP1 and SPA partially repress photomorphogenesis through PIF5.

(A-F) Phenotypic variations among seedlings of different genotypes, grown under either continuous dark (A) or red light (B). Box plots represent the mean hypocotyl length of seedlings growing in either dark (C) or continuous red light (D) and mean cotyledon opening angle of seedlings growing in either dark (E) or continuous red light (F). Surface sterilized seeds were plated onto MS medium without sucrose and incubated in either dark or red light for 4-day for seedling growth. Hypocotyl lengths and cotyledon angles were measured from more than 30 seedlings (n>30). Statistical significance among different genotypes was determined using one way ANOVA and Tukey's HSD tests, and is indicated by different letters. White bar = 10mm.

(G) Bar graph showing the anthocyanin content among seedlings of different genotypes. Two-day-old dark-grown seedlings were incubated in either dark or continuous white light for another two days. Crude samples of anthocyanin were prepared and measured as described in materials and methods. Statistical significance among different genotypes was determined using single factor ANOVA and Tukey's HSD tests, and is indicated by different letters.

Figure 5. Photoperiodic control of hypocotyl growth by PIF5 and COP1-SPA complex.

(A) Hypocotyl length of seedlings of different genotypes, growing under short-day (SD) condition. (B) Box plot showing measurement of hypocotyl length of seedlings shown in (A). Surface sterilized seeds were plated onto MS medium without sucrose and incubated in SD (8h light/16h dark) for 4-day. Statistical significance among different genotypes was determined using one way ANOVA and Tukey's HSD tests, and is indicated by different letters. White Bar=10mm.

(C) Rate of growth of hypocotyls from seedlings of different genotypes, growing under SD conditions. Surface sterilized seeds were plated onto MS medium without sucrose and incubated in SD (8h light/16h dark) for 4 days. Starting from day 2, infrared images were acquired every 1 hr and images were analyzed using ImageJ tool. Mean and SD were calculated and plotted into graphs.

Figure 6. COP1-SPA complex regulates diurnal oscillation of PIF5 protein level

(A, B) Immunoblots showing the diurnal oscillation pattern of PIF5-Myc protein level in *cop1-4* (A) and *spaQ* (B) mutants, compared to the wild-type seedlings. (C) Line graph showing diurnal oscillation of PIF5-Myc protein abundance in Col-0, *cop1-4* and *spaQ* background. Seedlings were grown under SD conditions (8h light/ 16h dark) for 3 days and samples were collected on 4^{th} day at the specific time ZT (h). Total protein was isolated and separated on 6.5% gel. Proteins were transferred to PVDF membrane and were sequentially blotted with anti-Myc and anti-RPT5 antibodies. For line graph, band intensities of PIF5-Myc and RPT5 from three replicates were measured using ImageJ tool and PIF5-Myc/RPT5 ratio was calculated. PIF5-Myc level in Col-0 at ZT4 was set to 1 and corresponding values were determined.

(D-F) Relative expression level of *PIF5* (D)*, ATHB2* (E) and *IAA29* (F) in 4-day-old SD-grown seedlings. Seedlings were grown under SD conditions (8h light/ 16h dark) for 3 days and samples were collected on 4^{th} day at the specific time ZT (h). Total RNA was isolated and cDNA was prepared from it. qRT-PCR was performed using gene specific primers and *PP2A* was used as an internal control.