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Short title: Circadian clock regulates photoperiodic growth

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Pseudo Response Regulators regulate photoperiodic hypocotyl growth by repressing *PIF4/5* transcription

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One-sentence summary: Pseudo Response Regulator proteins and the Evening Complex transmit daylength information to regulate photoperiodic hypocotyl growth by directly repressing transcription of key growth regulators.

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Abstract

The circadian clock measures and conveys daylength information to control rhythmic hypocotyl growth in photoperiodic conditions to achieve optimal fitness, but it operates through largely unknown mechanisms. Here, we show that Pseudo Response Regulators (PRRs) coordinate with the Evening Complex (EC), a transcriptional repressor complex within clock core oscillator, to specifically regulate photoperiodic hypocotyl growth in *Arabidopsis thaliana*. Intriguingly, a distinct daylength could shift the expression phase and extend the expression duration of PRRs. Multiple lines of evidence further demonstrated that PRRs directly bound the promoters of *PHYTOCHROME-INTERACTING FACTOR4* (*PIF4*) and *PIF5* to repress their expression, hence PRRs act as transcriptional repressors of the positive growth regulatorss *PIF4* and *PIF5*. Importantly, mutation or truncation of the TIMING OF CAB EXPRESSION 1 (TOC1) DNA binding domain, without compromising its physical interaction with PIFs, still caused long hypocotyl growth under short days, highlighting the essential role of the PRRs-*PIFs* transcriptional module in photoperiodic hypocotyl growth. Finally, genetic analyses demonstrated that *PIF4* and *PIF5* are epistatic to *PRRs* in the regulation of photoperiodic hypocotyl growth. Collectively, we propose that, upon perceiving daylength information, PRRs cooperate with EC to directly repress *PIF4* and *PIF5* transcription together with their post-translational regulation on PIFs activities, thus forming a complex regulatory network to mediate circadian clock-regulated photoperiodic growth.

Introduction

Seedlings of terrestrial flowering plants display diel rhythmic growth upon responding to recurring natural stimuli immediately after protruding from the soil. The photoperiod, i.e., the daylength, is the most prominent environmental factor that shapes plant architecture and determines growth phase transition. Photoperiod information, which reflects seasonal changes, can be processed by circadian clock-dependent mechanisms to shape the gene expression pattern, with an acrophase at a specific time of the day, thus to modulate a wide range of plant growth and developmental processes, including flowering time (Yanovsky and Kay, 2002; Valverde et al., 2004; Sawa et al., 2007; Sawa and Kay, 2011; Andres and Coupland, 2012; Lee et al., 2017). In particular, the seedling hypocotyl displays robust growth rhythms under certain photoperiodic conditions. The length of the hypocotyl is reversely associated with daylength, which has long been considered as a coordinative mechanism between the circadian clock and daily photoreception (Nozue et al., 2007; Niwa et al., 2009; Nomoto et al., 2012). Nevertheless, the regulatory network underlying this coordinative mechanism is largely unknown.

Phytochrome-interacting factors (PIFs), a group of basic helix–loop–helix transcription factors (Huq and Quail, 2002), can profile the hypocotyl photoperiodic growth dynamics, and are regarded as converging regulators to explain the coincidence between external environmental cues and the circadian clock (Millar, 2016; Quint et al., 2016). Under photoperiodic conditions, the protein abundance and activity of *PIFs*, especially of *PIF4* and *PIF5*, are concurrently regulated by light signaling and the circadian clock via a combination of transcriptional and post-transcriptional mechanisms (Fujimori et al., 2004; Shen et al., 2007; Nusinow et al., 2011; Nakamichi et al., 2012; Nieto et al., 2015; Soy et al., 2016; Zhu et al., 2016; Martin et al., 2018). Light signals modulate PIF protein abundance by triggering physical interaction between PIFs and phytochromes and subsequent degradation of PIFs (Al-Sady et al., 2006; Shen et al., 2007), while the circadian clock mainly shapes the circadian transcriptional waves of *PIF4* and *PIF5* (Nusinow et al., 2011; Nakamichi et al., 2012; Nieto et al., 2015; Soy et al., 2016; Zhu et al., 2016; Martin et al., 2018). Thus, the diurnal regulation of *PIF4* and *PIF5* transcription plays a critical role in photoperiodic hypocotyl cell elongation.

The circadian clock Evening Complex (EC), which is composed of EARLY FLOWERING 4 (ELF4), EARLY FLOWERING 3 (ELF3), and LUX ARRHYTHMO (LUX), inhibits *PIF4* and *PIF5* expression in the early evening and the first part of night, thus directly allowing the circadian clock to diurnally regulate hypocotyl growth (Nusinow et al., 2011). As the transcriptional peak phase of *PIF5* is ahead of *PIF4* for about 2–4 h, when EC proteins have not yet highly accumulated, it raises a possibility that other clock components are also involved in the progressive repression of *PIF4* and *PIF5*. Hence, the intricate regulation of *PIF4* and *PIF5* transcription remains to be fully unraveled (Nusinow et al., 2011; Nakamichi et al., 2012; Liu et al., 2013; Liu et al., 2016; Zhu et al., 2016; Martin et al., 2018).

The *Arabidopsis thaliana* Pseudo Response Regulator (PRR) gene family is composed of five members (*PRR9*, *PRR7*, *PRR5*, *PRR3*, and *TIMING OF CAB EXPRESSION 1* (*TOC1*)), each of which peaks at a specific time of day in a consecutive manner from dawn to dusk (Matsushika et al., 2000; Nakamichi et al., 2010). PRR proteins were proposed to regulate photoperiodic hypocotyl elongation mainly via two pathways. One is the transcriptional regulation of *PIF4* and *PIF5* by PRR5 and PRR7 (Liu et al., 2013; Nakamichi et al., 2012), and the other is the transcriptional activation activities of PIFs which are tightly regulated by the circadian clock via physical interaction between PIFs and PRRs (Soy et al., 2016; Zhu et al., 2016; Martin et al., 2018). Currently, the underlying mechanisms of the long hypocotyl phenotype of *prr* mutants in short-day (SD) conditions or in response to temperature are thought to be mainly due to their post-transcriptional regulation of PIFs via physical interactions and antagonistically with PIFs by binding to a set of co-targets, including *PHYTOCROME INTERACTING FACTOR 3-LIKE 1* (*PIL1*), *YUCCA 8* (*YUC8*), and *CYCLING DOF FACTOR 5* (*CDF5*) (Martin et al., 2018; Soy et al., 2016; Zhu et al., 2016). In addition, TOC1 can physically interact with ELF3 (Huang et al., 2016), the bridging protein among EC, but it is still unclear whether ELF3 and TOC1 work in the same pathway or independently to regulate photoperiodic hypocotyl growth. Moreover, how PRRs respond to distinct daylength information at the transcriptional and post-transcriptional level and

subsequently transmit photoperiod information to control hypocotyl cell elongation is still largely unknown.

Here, we show that PRRs and EC act additively in regulating photoperiodic hypocotyl growth in Arabidopsis, and daylength information can alter the expression phase and duration of PRRs. We further unveiled *PIF4* and *PIF5* as direct transcriptional targets of PRRs, and their transcriptional patterns were accordingly altered by daylength information via PRRs. Importantly, by using TOC1 DNA binding domain mutation or truncation alleles, we show that the PRRs-*PIF*s transcription module is essential for regulating hypocotyl growth in photoperiodic conditions. Together with the post-translational regulation of PIF abundance and activities by PRRs and EC, we thus propose a complex regulatory network that mediates circadian clock-regulated photoperiodic hypocotyl growth, by a combinatorial transcriptional and post-transcriptional mechanisms.

Results

PRRs Act Additively with EC to Regulate Photoperiodic Hypocotyl Growth

Both PRRs and EC are involved in hypocotyl growth regulation (Sato et al., 2002; Kaczorowski and Quail, 2003; Yamamoto et al., 2003; Nusinow et al., 2011; Nieto et al., 2015; Soy et al., 2016; Zhu et al., 2016; Martin et al., 2018, Li et al., 2019). TOC1, the founding member of PRRs, can physically interact with an EC component ELF3 (Huang et al., 2016). Nevertheless, the relationship between PRRs and EC in regulating hypocotyl growth, especially under photoperiodic conditions, is unclear. To systematically address this question, we generated higher order Arabidopsis mutants between PRRs and EC components. After growth for 5 days at different conditions, we measured the hypocotyl length and found that *toc1*, *prr5*, *toc1 prr5*, and *elf3* mutants displayed dramatically longer hypocotyl phenotypes under both short-day (SD, 8 h light / 6 h dark) and long-day (LD, 16 h light / 8 h dark) conditions relative to Col-0, but not under constant light (LL) conditions (Figure 1A-1F). Strikingly, the hypocotyls of *toc1 elf3* and *prr5 elf3* double mutants were significantly longer than those of the single mutants, suggesting that they act additively to regulate hypocotyl

growth only under photoperiod conditions. Notably, the hypocotyl lengths of the *toc1 prr5 elf3* triple mutant were modestly but significantly longer than those of the *toc1 prr5* and *elf3* mutants under both LD and SD conditions ((Fig. 1A-1D), further supporting the notion that PRRs and EC additively regulate hypocotyl growth. Since ELF3 has been shown to interact with PIF4 to regulate hypocotyl growth independent of EC (Nieto et al., 2015), we further examined the genetic relationship between PRRs and EC by using LUX, a DNA binding protein in EC (Hazen et al., 2005; Nusinow et al., 2011). Consistently, the *toc1 prr5 lux* triple mutant displayed significantly longer hypocotyls than either the *toc1 prr5* or *lux* mutants, in both SD and LD conditions (Supplemental Fig. S1), further confirming that PRRs and EC additively regulate photoperiodic hypocotyl growth. In addition, the transcript phases of *PRR9* and *PRR7* displayed an inverse pattern to that of EC, but the hypocotyls of the *prr7 prr9* double mutant were significantly longer than that of Col-0 (Nakamichi et al., 2005), specifically under photoperiodic conditions, but not in constant light (Supplemental Fig. S2). Altogether, multiple lines of genetic evidence clearly demonstrated that PRRs act additively with EC to regulate hypocotyl growth under photoperiodic conditions.

Daylength Information Alters the Expression Patterns of PRRs and EC

In general, the hypocotyl length decreases with increasing daylength. However, the ratio of hypocotyl length in SD vs. LD conditions was significantly increased in the *toc1* mutant compared to that in *prr5*, *elf3*, or Col-0 plants (Fig. 1A-1D). This prompted us to compare the expression patterns of *TOC1* and other *PRR* family members under SD and LD conditions. Previously, it has been shown that the transcript and protein abundances of each *PRR* gene peaks sequentially from dawn to dusk in the order of *PRR9*, *PRR7*, *PRR5*, *PRR3* and *TOC1* (Matsushika et al., 2000; Fujiwara et al., 2008; Martin et al., 2018). However, whether the distinct daylength information could change their mRNA or protein patterns remains unclear. By using a time-course reverse transcription quantitative PCR (RT-qPCR) assay and the publicly accessible database, we found that the expression pattern of *TOC1* was overall shifted by about 4 h in SD vs. LD conditions, while the *PRR5* mRNA expression pattern was not significantly altered by the daylength difference (Supplemental Fig. S3 and Supplemental Fig.

S4). Interestingly, when we compared the protein expression patterns of TOC1 and PRR5 between SD and LD conditions by using previously generated *TMG* (*TOC1* Mini Gene driven by its native promoter) and *PRR5pro:PRR5-GFP* transgenic lines (Mas et al., 2003; Fujiwara et al., 2008), we found that the duration and peak times of TOC1 and PRR5 proteins are highly variable under these two distinct conditions. This might have been caused by post-transcriptional regulation, given that the *PRR5* mRNA pattern did not display a phase shift. Moreover, both PRR5 and TOC1 proteins were barely detectable at ZT20 in SD conditions, but were still present in an appreciable level at ZT20 in LD conditions (Fig. 2A-2D). Remarkably, the high TOC1 protein level could even extend to ZT0 in the night under LD conditions (Fig. 2A, 2B). In addition, the protein abundance of two other PRR family members, PRR9 and PRR7, started to rise from ZT4, and persisted over the day time in both LD and SD conditions. The PRR7 protein was maintained at a higher level with increasing daylength (Fig. 2E-2H). Interestingly, among EC components, transcripts of *LUX* and *ELF4* displayed a similar shifted pattern as *TOC1* in SD conditions, while *ELF3* only showed an increased expression level without pattern shifting in SD conditions (Supplemental Fig. S4E-4G). Thus, it appeared that the daylength information could either shift the expression phase or extend the expression period of PRRs and EC at both the transcriptional and post-transcriptional levels, which might contribute to the daylength-dependent photoperiodic hypocotyl growth.

***PIF4* and *PIF5* are Potential Common Transcriptional Targets of PRRs and EC**

To further elucidate the underlying mechanisms of how PRRs coordinate with EC to regulate photoperiodic hypocotyl growth, we identified their direct transcriptional targets, as both of them are transcription regulators (Gendron et al., 2012; Huang et al., 2012; Nakamichi et al., 2012). RNA-sequencing (RNA-seq) with 10-day old seedlings of *toc1 prr5* grown under 12 h light/12 h dark conditions was conducted with tissues harvested at ZT15; the exact same time point used for TOC1 ChIP-seq (Huang et al., 2012) and close to the time point for PRR5 ChIP-seq (Nakamichi et al., 2012). In total, we identified 838 differentially expressed genes (DEGs) in the *toc1 prr5* double mutant using 2-fold cut-off (FDR<0.05) compared to Col-0

183 (Fig. 3A, Supplemental Dataset 1). The randomly selected 4 up-regulated genes and 4
184 down-regulated genes validated by RT-qPCR displayed similar expression patterns as that in
185 the RNA-seq data (Supplemental Fig. S5). Notably, *CIRCADIAN CLOCK ASSOCIATED 1*
186 (*CCA1*), *LATE ELONGATED HYPOCOTYL* (*LHY*), and *GIGANTEA* (*GI*), and some other
187 core circadian clock genes, were among the 270 up-regulated genes, consistent with the fact
188 that they are direct targets of TOC1 within the interlocked circadian clock oscillator (Huang et
189 al., 2012). Functional assignment of the DEGs by gene ontology (GO) enrichment analysis
190 further revealed that the DEGs were mainly involved in response to red or far-red light,
191 response to light stimulus, circadian rhythms, and red/far-red light photo-transduction (Fig.
192 3B), implicating a dual role for TOC1 and PRR5 in regulating the circadian clock and light
193 signaling. Among them, we found that transcript levels of *PIF4* and *PIF5* were significantly
194 increased in the *toc1 prr5* mutant (Fig. 3C). Previous ChIP-Seq analysis identified 772
195 TOC1-bound genes (Huang et al., 2012), 1021 PRR5-bound genes (Nakamichi et al., 2012),
196 and 1096 PRR7-bound genes (Liu et al., 2013). As the PRRs play redundant roles in regulating
197 photoperiodic hypocotyl growth, we thus compared the ChIP-seq data of PRR7, PRR5, and
198 TOC1, and obtained 90 commonly bound genes (Fig. 3D, Supplemental Fig. S6). The
199 interaction network analysis using the STRING database (http://string-db.org/) showed that the
200 90 common genes could form a major cluster, including known circadian clock genes, such as
201 *CCA1*, *LHY*, and *GI*, and genes involved in photomorphogenesis, including *PIF4*, *PIL6/PIF5*,
202 and *PHYTOCHROME B* (*PHYB*) (Fig. 3E). The potential direct target genes of PRRs were
203 further revealed by comparing our RNA-seq data with the PRR7/PRR5/TOC1 common target
204 genes. Strikingly, *PIF4* and *PIF5* were found among the 11 overlapping genes (Supplemental
205 Fig. S6, hypergeometric test, $p < 3.5 \times 10^{-9}$) between up-regulated genes in the *toc1 prr5* mutant
206 and the 90 common target genes, indicating that *PIF4* and *PIF5* were potential direct target
207 genes of TOC1 and PRR5. Furthermore, when we compared the aforementioned 11 overlapped
208 genes with the up-regulated genes in the *lux-6* mutant, *PIF4* and *PIF5* were again among the
209 only 4 common co-targets (Fig. 3F, 3G). Hence, *PIF4* and *PIF5* became promising target genes
210 of EC and PRRs in mediating their regulation of photoperiodic hypocotyl growth.

PRRs directly Bind *PIF4* and *PIF5* Promoters to Repress Their Transcription

As *PIF4* and *PIF5* are two potential common transcriptional targets of PRRs and EC, we determined whether PRRs could directly repress *PIF4* and *PIF5* transcription. Promoter analysis suggested that one potential TOC1 and PRR5 binding element, *PIF4-G* (G-box, GATATG) (Gendron et al., 2012), was found at -707 bp upstream of the *PIF4* start codon, and two G-boxes, *PIF5-G1* (G-box, GATATG) and *PIF5-G2* (G-box, GATATG), are found at -1151 bp and -718 bp upstream of the *PIF5* start codon, respectively (Fig. 4A). We then conducted electrophoretic mobility shift assays (EMSA) with the purified GST-tagged CCT domain of TOC1 and PRR5, which is the DNA-binding domain of PRRs (Gendron et al., 2012). Both GST-TOC1-CCT and GST-PRR5-CCT could efficiently bind the *PIF4-G* and *PIF5-G2* regions compared to GST alone (Fig. 4B), as well as bind the *CCA1* promoter (as a positive control) (Supplemental Fig S7A), but not the *PIF5-G1* region. Importantly, the binding could be abolished by the non-labeled competitive probe, suggesting that TOC1 and PRR5 could specifically bind the promoters of *PIF4* and *PIF5* (Fig. 4B and Supplemental Fig. S7A). Results of ChIP-qPCR analysis further confirmed that the amplicons containing the *PIF4* promoter G-box and *PIF5* promoter G2 regions were significantly enriched in *TMG* lines ranging from ZT12 to ZT20 and in *PRR5:PRR5-GFP* from ZT8 to ZT16 (Fig. 4C, 4D), in line with the TMG and PRR5 protein expression window. Similar binding enrichment was observed for the amplicons for the *CCA1* promoter, but not the negative control *ASCORBATE PEROXIDASE 3* (*APX3)* (Supplemental Fig. S7B, 7C). These results are consistent with previous ChIP-seq studies (Huang et al., 2012, Nakamichi et al., 2012). Taken together, TOC1 and PRR5 could directly bind *PIF4* and *PIF5* promoters *in vitro* and *in vivo*.

Whether TOC1 and PRR5 could directly repress *PIF4* and *PIF5* transcription was determined by monitoring the bioluminescence signals of *PIF4pro:LUC* and *PIF5pro:LUC* using well-established transient expression systems in the leaves of *Nicotiana benthamiana* and in Arabidopsis protoplast. Results of the transient expression analyses clearly indicated that the transcriptional activities of *PIF4* and *PIF5* could be repressed by PRRs (Fig. 4E-4H and

Supplemental Fig. S8). Collectively, our results supported the notion that *PIF4* and *PIF5* are direct transcriptional targets of PRRs.

PRRs Cooperate with EC in Timing Photoperiodic Transcription of *PIF4* and *PIF5*

As *PIF4* and *PIF5* are the common transcriptional targets of PRRs and EC, and daylength could alter the expression patterns of PRRs and EC, we questioned whether PRR proteins could coordinate with EC in conveying daylength information to control photoperiodic hypocotyl growth through the timing of *PIF4* and *PIF5* transcription. To test this, *PIF5pro:PIF5-HA* transgenic plants were generated to investigate the temporal protein pattern of PIF5 under SD and LD conditions. Intriguingly, the PIF5 protein abundance was inversely associated with TOC1 and PRR5 protein abundance (Fig. 2A-2D) under both SD (Fig. 5A) and LD (Fig. 5B) conditions, consistent with the idea that TOC1 and PRR5 directly repressed *PIF5* transcription. Similarly, PIF4 protein has been observed to accumulate during the light period and decrease in the dark period from ZT12 to ZT20, then increase before dawn under a short day but not under a 12 h light/12 h dark photoperiod. As PIF4 and PIF5 protein accumulation was associated well with their transcription, *PIF4* and *PIF5* transcript levels were examined in the *toc1 prr5* double mutant and *toc1 prr5 elf3* triple mutant. Results of RT-qPCR indicated that *PIF4* and *PIF5* transcript levels were similar to that of Col-0 at the subjective day time in both *toc1 prr5* and *toc1 prr5 elf3* mutants, but modestly increased at the subjective early night, and more significantly accrued at late night, especially at ZT20 in both photoperiodic conditions (Fig. 5C-5F). As EC represses *PIF4* and *PIF5* transcription from dusk to early night, *PIF4* and *PIF5* transcript levels displayed a modest but consistent increase in the *toc1 prr5 elf3* triple mutant compared to those in *toc1 prr5* or *elf3* mutants, especially under LD conditions (Fig. 5C-5F). Similarly, the transcript levels of *PIF4* and *PIF5* were also significantly elevated in *prr7 prr9* and *prr5 prr7 prr9* mutants under both SD and LD conditions (Supplemental Fig. S9). Together, our results support a notion that PRRs in concert with EC repress the transcription of *PIF4* and *PIF5*, hence to shape their transcriptional patterns in mediating circadian clock-regulated photoperiodic hypocotyl growth.

Direct Transcriptional Inhibition of *PIF4* and *PIF5* by TOC1 is Required for its Regulation of Photoperiodic Hypocotyl Growth

As the physical interaction of PRRs with PIFs antagonizes PIFs function under a diurnal cycle (Martin et al., 2018; Soy et al., 2016; Zhu et al., 2016), a truncated TOC1 without the CCT DNA-binding domain (Gendron et al., 2012) was used to test if PRR-mediated *PIF4/5* repression was required in photoperiodic hypocotyl growth. Similar to the full-length TOC1, GFP-TOC1ΔCCT-NLS was predominantly localized in nuclear speckles both in the epidermal cells of infiltrated *N. benthamiana* leaves and in the hypocotyl cells of stable transgenic Arabidopsis plants (Supplemental Fig. S10). Importantly, the truncated TOC1 protein without its DNA binding domain could still physically interact with PIF4 and PIF5, with a similar affinity as full-length TOC1 (Fig. 6A and Supplemental Fig. S11A), as the CCT domain was dispensable in mediating TOC1-PIFs interaction in yeast (Zhu et al., 2016). However, the transcriptional repression of *PIF4* and *PIF5* by the truncated TOC1 protein without its CCT domain was severely compromised compared to the full-length TOC1 (Supplemental Fig. S12). Notably, overexpression of full-length TOC1, but not TOC1ΔCCT, could fully rescue the long hypocotyl phenotype of the *toc1-21* mutant grown in SD conditions, even when the *TOC1* ectopic expression levels were comparable or lower than the endogenous *TOC1* (Fig. 6B). Consistently, the transcript levels of *PIF4* and *PIF5* were significantly repressed by overexpression of full-length *TOC1* but not *TOC1ΔCCT* (Fig. 6C). Compared to that in *toc1-21* mutants, the moderately shortened hypocotyl phenotypes in the *TOC1ΔCCT* transgenic lines was likely due to TOC1ΔCCT-PIFs interaction and sequestration of PIF function (Martin et al., 2018; Soy et al., 2016; Zhu et al., 2016).

A missense allele of *toc1-1* caused by an A562V mutation in the TOC1 DNA binding domain (Strayer et al., 2000) was further employed to distinguish the direct transcriptional role of TOC1 on *PIF4* and *PIF5* from its post-translational regulation of PIFs via sequestration. Similar to TOC1ΔCCT, the TOC1 A562V protein could still physically interact with PIF4 and PIF5 like the wild-type TOC1 (Fig. 6D and Supplemental Fig. 11B). However, the TOC1 A562V had much reduced ability to bind *PIF4* and *PIF5* promoters in the EMSA (Fig. 6E),

similar to the results of a previous report on the binding of the *CCA1* promoter by TOC1 A562V (Gendron et al., 2012). As the *toc1-1* mutant still displayed long hypocotyl phenotypes (Dowson-Day and Millar, 1999) under SD conditions (Fig. 6F), it further supported the idea that the TOC1-*PIFs* transcriptional module played a pivotal role in regulating photoperiodic hypocotyl growth.

***PIF4* and *PIF5* are Epistatic to *PRRs* in Regulating Photoperiodic Hypocotyl Growth**

As *PIF4* and *PIF5* are direct PRR transcriptional targets, together with the PRR physical interaction with PIFs to sequester their activity (Martin et al., 2018; Soy et al., 2016; Zhu et al., 2016), we proposed that PIF4 and PIF5 act as major downstream factors to mediate circadian clock-regulated photoperiodic hypocotyl growth. Thus, we determined if *PIF4* and *PIF5* were required for PRR-mediated circadian clock regulation of hypocotyl elongation by generating a variety of higher order mutants. In agreement with a previous report (Soy et al., 2016), the long hypocotyl phenotypes in *toc1* and *toc1 prr5* mutants could be partially reverted by a single introgression of *pif4* under either LD or SD conditions. Moreover, the long hypocotyl phenotype in the *toc1 prr5* mutant could be completely rescued to the wild-type (Col-0) level by an introgression of *pif4 pif5* mutations under either LD or SD conditions (Fig. 7A-7D), indicating a redundancy of *PIF4* and *PIF5* in meditating photoperiodic hypocotyl growth. The hypocotyl length in various mutants including *toc1*, *toc1 pif4*, *pif4*, *toc1 prr5*, *pif4 pif5*, *toc1 prr5 pif4*, and *toc1 prr5 pif4 pif5*, were indistinguishable from that of Col-0 under continuous light conditions (Fig. 7E-7F), further reinforcing the notion that the repression of *PIF4* and *PIF5* by PRRs at both the transcriptional and post-transcriptional levels is required to concurrently regulate photoperiodic hypocotyl growth by the circadian clock. Given a previous report showing that mutations of PIF4 and PIF5 inhibit the long hypocotyls of *prr* mutants (Martin et al., 2018; Soy et al., 2016) under SD conditions, our evidence further demonstrates that *PIF4* and *PIF5* function downstream of PRRs to mediate photoperiodic hypocotyl growth.

Discussion

By sensing photoperiod, the plant circadian clock regulates a plethora of daily rhythmic physiological events (Yanovsky and Kay, 2002; Valverde et al., 2004; Sanchez and Kay, 2016). The hypocotyl displays a robust rhythmic elongation pattern under photoperiodic conditions by a coincidental mechanism between the circadian clock and external light signals (Nozue et al., 2007; Niwa et al., 2009; Nomoto et al., 2012). Nevertheless, how the circadian clock coordinates with the external photoperiod to facilitate optimal hypocotyl growth remains largely unknown. *PIF4* and *PIF5* have been characterized as potential targets of PRR5 and PRR7 (Liu et al., 2013; Nakamichi et al., 2012). However, the temporal transcriptional regulation of PRR proteins to *PIF4* and *PIF5*, especially under distinct photoperiodic cycles, are still largely unclear. In this study, we found that PRRs genetically act additively with EC to regulate photoperiodic hypocotyl growth. We further demonstrated that PRRs directly bound the promoters of *PIF4* and *PIF5* to repress their transcription, and the altered temporal patterns of PRRs by daylength information could subsequently change *PIF4* and *PIF5* mRNA expression patterns, thus mediating photoperiodic hypocotyl growth (Fig. 8). By using specific TOC1 alleles, our results unequivocally showed that the transcriptional regulation of *PIF4* and *PIF5* is critical for PRR-regulated photoperiodic hypocotyl growth. In addition to post-translational regulation of PIF abundance and activities by PRRs and ELF3 (Martin et al., 2018; Nieto et al., 2015; Soy et al., 2016; Zhu et al., 2016), here we show that PRRs cooperate with EC to control *PIF4* and *PIF5* temporal transcription patterns which mediates the crosstalk between the circadian clock and light signaling to achieve optimal hypocotyl growth and fitness under photoperiodic conditions.

Sensing and transmitting daylength information has long been proposed as an interplay between the circadian clock and external photoperiod, with mainly unclear mechanisms. Hypocotyls displays diel rhythmic growth patterns after emerging from the soil in natural photoperiodic conditions, but the underlying molecular mechanism remains unclear. Differential daylength information, i.e., a long day vs. short day, can drastically change the expression pattern and period of *PRR* transcripts and proteins, indicating that daylength information can be transmitted at least through PRRs and EC via both transcriptional and

post-transcriptional mechanisms. The altered expression pattern of PRRs, particularly for TOC1 and PRR5, subsequently causes altered expression of *PIF4* and *PIF5* transcripts and proteins, hence to affect daylength-dependent hypocotyl growth patterns (Fig. 5). The reason why PRRs and EC act additively on the regulation of *PIF4* and *PIF5* transcription could be explained by their differential binding sites within the *PIF4* and *PIF5* promoters, but not due to the physical interaction between TOC1 and ELF3 (Huang et al., 2016). Hence, the biological significance of TOC1 physically interacting with ELF3 awaits to be further explored. Intriguingly, daylength information does not alter either the transcript level or expression pattern of *PRR5* (Supplemental Fig. S3B), but the overall expression pattern of PRR5 protein was shifted by about 4 h earlier in SD conditions (Fig. 2C, 2D), indicating that daylength information sensing and transmission also occurs at the post-transcriptional level for photoperiodic hypocotyl growth. A similar case has been observed for photoperiod-regulated flowering time in which the CONSTANS (CO) protein level is tightly controlled by a coincident mechanism between the circadian clock and photoperiod (Valverde et al., 2004; Song et al., 2012). It will be of great interest to decipher how daylength information affects the expression patterns of PRRs in future studies.

The expression of *PIF4* and *PIF5* oscillates with a peak after dawn, and then decreases gradually (Nusinow et al., 2011). EC represses the expression of *PIF4* and *PIF5* at nighttime, but aside from EC, how *PIF4* and *PIF5* are regulated by other circadian clock components at the transcriptional level is still not clear. Our present findings here filled this knowledge gap, and we proposed that, in LD conditions, the extended expression time-frame and the shifted expression pattern together maximize the repression of PRRs on *PIFs* expression, thus inhibiting hypocotyl growth. While in SD conditions, PRR5 and TOC1 proteins do not accumulate before the subjective dawn range from ZT20 to ZT24, which causes high abundance of *PIF4* and *PIF5* to promote hypocotyl growth. Taken together, our findings revealed a key underlying mechanism by which the PRRs-*PIF4/5* transcriptional module finely orchestrates circadian photoperiodic responsive hypocotyl growth in Arabidopsis.

Very recently, CCA1 and LHY, the two morning-phased circadian core components, were shown to recruit SHORT HYPOCOTYL UNDER BLUE 1 (SHB1) to promote *PIF4* transcription by directly binding to the *PIF4* promoter (Sun et al., 2019). Our EMSA results (Fig. 3b, 5e and Supplemental Fig. 7) and previous evidence clearly demonstrated that PRRs can bind the G-box cis-elements of *CCA1*, *PIF4*, and *PIF5* promoters to repress their transcription. Collectively, the transcription of *PIF4* and *PIF5* was intricately modulated by the circadian clock, among which CCA1 and LHY act as daytime transcriptional activators, while PRRs and EC cooperatively act as transcription repressors to sequentially repress *PIF4* and *PIF5* transcription (Fig. 6c). Meanwhile, PRRs and ELF3 also inhibit PIFs' activities at the post-translational level by physically interacting with PIF proteins. Together, the complex regulatory network, integrating both transcriptional and post-transcriptional regulation of PRRs and EC on PIFs, collectively limits the function of PIFs from morning to early evening, to precisely time the higher growth rate in the late night. Intriguingly, GI, another key circadian clock protein, was recently reported to play a pivotal role in modulating light signaling through physical interaction with PIFs (Nohales et al., 2019). GI protein not only negatively regulates PIFs' protein stabilities, but also occupies PIFs' genomic target loci in the early evening (Nohales et al., 2019). Hence, it is conceivable that the circadian clock tightly coordinates photoperiodic hypocotyl growth by integrating multiple circadian regulation mechanisms on PIFs at both the transcriptional and post-transcriptional levels. As PIF4 and PIF5 serve as a central cellular signaling hub by integrating phytohormones, light signaling, and circadian signals to control many downstream physiological processes, such as senescence (Song et al., 2014; Nohales et al., 2019), shade avoidance and temperature signaling (Ma et al., 2016; Pedmale et al., 2016), it will be of great interest in the future to investigate whether the PRRs-*PIF4/5* transcriptional module plays other roles besides photoperiodic hypocotyl growth control.

Materials and Methods

Plant materials and growth conditions. Except where indicated, all of the *Arabidopsis thaliana* plants used in this study were in the Col-0 background, including WT, *toc1-21* (Ding

et al., 2007), *prr5-1* (Wang et al., 2010), *prr5-1 prr7-11* (Yamashino et al., 2008), *prr5-1 prr9-10* (Yamashino et al., 2008), *prr7-11 prr9-10* (Yamashino et al., 2008), *prr5-1 prr7-11 prr9-10* (Yamashino et al., 2008), *elf3-1*(Nusinow et al., 2011), *lux-6* (Zhang et al., 2018), *TMG* (Mas et al., 2003), *PRR5pro:PRR5-GFP* (Fujiwara et al., 2008), *PRR7pro:PRR7-GFP* (Fujiwara et al., 2008), *PRR9pro:PRR9-GFP* (Fujiwara et al., 2008), *pif4-2* (Leivar et al., 2008), *pif4-2 pif5-3* (CS68096). *toc1-21 prr5-1*, *toc1-21 elf3-1*, *prr5-1 elf3-1*, *toc1-21 prr5-1 elf3-1*, *toc1-21 prr5-1 lux-6*, *toc1-21 pif4-2*, *toc1-21 prr5-1 pif4-2*, and *toc1-21 prr5-1 pif4-2 pif5-3* were generated by crossing. The sterilized Arabidopsis seeds were stratified at 4°C for 3 days, and then transferred to a 22°C growth chamber with light/dark cycles of 12 h light/12 h dark, 16 h light/8 h dark, or 8 h light/16 h dark as indicated.

Plasmids construction. For the transient transcriptional repression assays in *Nicotiana benthamiana*, the amplicons of *PIF4* and *PIF5* promoters from about 2000 base pairs upstream of their start codons were amplified from Col-0 genomic DNA, then were inserted into the promoter-free *pLUC-N-1300* vector between the *Pst* I and *Kpn* I sites to generate the *PIF4pro:LUC-N-1300* and *PIF5pro:LUC-N-1300* constructs, respectively. To prepare the vectors of *PIF4pro:LUC* and *PIF5pro:LUC* for Arabidopsis protoplast transient expression analysis, the same sequences of *PIF4* and *PIF5* promoters were digested with *Bam*H I and *Bsu*36 I, and then cloned into the *pLUC-999* vector.

Hypocotyl length measurements. Sterilized seeds were placed on MS medium (PhytoTech, M524) for 3 days of incubation at 4°C, then incubated in specific light photoperiod conditions (12 h light/12 h dark cycles, 16 h light/8 h dark, or 8 h light/16 h dark; white light: 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$, Digital light meter, TES-1332A) for 5 additional days. Seedlings were photographed and hypocotyl lengths were measured by using Image *J* software (http://rsb.info.nih.gov/ij).

Protein detection method for PRRs. Seedlings of *TMG, PRR5pro:PRR5-GFP, PRR7pro:PRR7-GFP* and *PRR9pro:PRR9-GFP* transgenic lines were grown under SD or LD conditions (8 h light/16 h dark, or 16 h light/8 h dark; light intensity: 200μmol·m^{-2}·s^{-1}, Digital light meter, TES-1332A) for 10 days, and samples were harvested in 4-h intervals during a 24-hour cycle. Total proteins were extracted with IP buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40 (v/v), 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/mL leupeptin, 1 μg/mL aprotinin, 1 μg/mL pepstatin, 5 μg/mL antipain, 5 μg/mL chymostatin, 2 mM $NaVO_3$, 2 mM NaF, 50 μM MG132, 50 μM MG115, 50 μM ALLN). Supernatants were resolved using an 8% SDS-PAGE gel. The respective proteins were detected by western blotting using GFP antibody (Abcam; ab6556).

RNA-sequencing analysis. For the RNA-seq assays, plants were grown under 12 h light / 12 h dark conditions at 22°C for 10 days and harvested at ZT15. RNA-sequencing and differential gene expression analyses were performed at Bionova (Beijing, China). In brief, RNA quality was evaluated on a Bioanalyzer 2100 instrument (Agilent, Santa Clara, CA). Sequencing libraries were prepared following the protocol of the Directional RNA Library Prep Kit (NEB #E7760S). The 150 nt paired-end high-throughput sequencing was performed on an Illumina Hiseq X TEN. Low quality sequencing reads were removed. Clean reads were mapped to the Arabidopsis reference genome (TAIR10, www.arabidopsis.org) with Tophat2 (https://ccb.jhu.edu/software/tophat/index.shtml) software, and differentially expressed genes (DEGs) were identified using edgeR in the R package (http://www.bioconductor.org/packages/release/bioc/html/edgeR.html) with Fold Change > 2 and FDR < 0.05 between the case group sample and control group sample. Gene ontology (GO) enrichment analysis was performed using TopGO in the R package (http://bioconductor.org/).

Reverse Transcription Quantitative PCR for gene expression analysis. Seedlings were grown under specific light photoperiod conditions (12 h light/12 h dark, 16 h light/8 h dark, or 8 h light/16 h dark; light intensity: 200 μmol m^2s^{-1}) for 10 days, and samples were harvested in

4-h intervals during a 24-h period. Total RNA was extracted using TRIzol Reagent (Life Technologies) as described by the manual. One microgram RNA was used for reverse transcription with the PrimeScript RT Reagent Kit with gDNA Eraser (Takara). Quantitative PCR was performed using SYBR Green Real-Time PCR Master Mix (Toyobo, Osaka, Japan) according to the manufacturer's instructions on a QuantStudio 3 instrument (Applied Biosystems, USA). The following PCR program was used: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 15 s, followed by a melting-curve analysis. Gene expression was normalized by the geometric mean of *ACTIN2* and *TUB4* expression as previously described (Li et al., 2019). Experiments were repeated with at least two biological and two technical replicates. Data represent means ± SD of two technical replicates. Primers used for quantitative PCR are listed in Supplemental Table 1.

Transient transcriptional repression activity assay in *N. benthamiana*. *Agrobacterium tumefaciens AGL* carrying various fusion expression vectors (effector: *GFP-TOC1*, *GFP-PRR5*, *GFP-PRR7*, *GFP-PRR9*, or *GFP*; reporter: *PIF4pro: LUC-1300*, *PIF5pro: LUC-1300*, and *CCA1pro: LUC-1300*) were cultured overnight. Each reporter vector paired with the *GFP-TOC1*, *GFP-PRR5*, *GFP-PRR7*, *GFP-PRR9*, or *GFP* effector vector was then co-transformed into *N. benthamiana* leaves using a syringe infiltration method. The luciferase signal was detected using a CCD camera (LN/1300-EB/1, Princeton Instruments) 2 days after infiltration. The bioluminescence intensity of LUC signals was quantified by MetaMorph Microscopy Automation and Image Analysis Software (Molecular Devices, San Jose, United States).

Arabidopsis protoplast transient expression analysis. Protoplasts were isolated from rosette leaves of four-week old Arabidopsis plants (Col-0). For transient expression assays, 200 μL of protoplast was transferred to a 2 mL microfuge tube containing 5 μg effector plasmid, 3 μg reporter plasmid, and 2 μg *35S::GUS* plasmid which was used as an internal control. The

effector:reporter:GUS were co-transformed into protoplasts at a ratio of 5:3:2., and the LUC/GUS ratio was presented as normalized gene expression. *PIF4pro:LUC-1300*, *PIF5pro:LUC-1300*, and *CCA1pro:LUC-1300* were used as reporters, and *35S:GFP-TOC1*, *35S:GFP-PRR5*, *35S:GFP-PRR7*, *35S:GFP-PRR9*, and *35S: GFP* were used as effectors. The protoplasts were incubated for 16–24 h at 22°C. The luminescence measurements were acquired with a luciferase assay system (Promega, E1500) on a GloMax 20/20 luminometer (Promega). The GUS activity was detected with 4-Methylumbelliferone glucuronide (MUG) substrate (Alfa) on a GloMax 20/20 luminometer.

Chromatin Immunoprecipitation (ChIP) assays. ChIP assays were performed using *TMG* and *PRR5pro:PRR5-GFP* transgenic lines grown under 22°C in a growth chamber with 12 h light/12 h dark cycles for two weeks, and seedlings were harvested at 4-h intervals during a 24-h period (ZT0, ZT4, ZT8, ZT12, ZT16, and ZT20) as noted. ChIP experiments were performed as described (Huang et al., 2012). GFP antibody (Invitrogen; ab11120) was used for immunoprecipitation. The immunoprecipitates were analyzed by qPCR. Data are presented as mean ± SD, n = 3 from biological replicates. Primers used in this assay are shown in Supplemental Table 1.

Purified GST-tagged CCT domain of TOC1 and PRR5 proteins. *GST-TOC1* or *PRR5-CCT* plasmids were transformed into *Escherichia coli* BL21 strain, induced with 1 mM IPTG and cultured overnight at 16°C. The cells were collected by centrifuging at 10,000 rpm for 10 minutes, then the cells were resuspended in 10 mL extraction buffer (50 mM Tris-Cl, pH 8.0, 250 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 μg/mL leupeptin, 1 μg/mL aprotinin, 1 μg/mL pepstatin). Lysozyme was added and the reaction was incubated on ice for 30 minutes, then 100 μL 1M DTT and 1 mL 10% sarkosyl (w/v) were added and thoroughly mixed. Then, the lysate was sonicated until it became transparent. 2.3 mL Triton-X-100 was added and mixed for five minutes. After centrifuging at 10,000 rpm for 10 minutes, the supernatant was incubated with 500 μL GST-resin at 4°C for 3 hours. The beads were washed with wash buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 3 mM

513 dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.5% Triton X-100 (v/v) for 5 times. The
514 GST-resin was eluted with a reduced glutathione solution to obtain a GST-TOC1 or
515 PRR5-CCT protein solution.
516
517 **EMSA.** The Lightshift Chemiluminescent EMSA kit (Thermo Scientific) was used for EMSA.
518 5 μL GST-TOC1-CCT, GST-PRR5-CCT or GST protein and 0.5 μL of each biotin-labeled
519 probe was used in all assays. Protein and probe were incubated in 1× Lightshift binding buffer,
520 0.05 μg/μL poly(dI-dC), 2.5% (vol/vol) glycerol, 0.05% Nonidet P-40 (v/v), 50 mM KCl, and 5
521 mM $MgCl_2$ for 1 h at 4°C. Six percent gels were used. Gel running, transfer, and imaging were
522 done as described by the Lightshift kit as previously described (Gendron et al., 2012).
523
524 **Co-immunoprecipitation assay.** Agrobacteria containing *35S::TOC1-GFP* or *TOC1 CCT*
525 domain deletions, *35S::PRR5-GFP* or *PRR5 CCT* domain deletions, and *CsVMV::PIF4-HA* or
526 *CsVMV::PIF5-HA* were co-infiltrated into 4-week-old *N. benthamiana* leaves. The infiltrated
527 leaves were ground to a fine powder in liquid nitrogen after infiltration for 3 days. Total protein
528 was extracted with ice-cold IP buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5% Nonidet
529 P-40 (v/v), 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/mL
530 leupeptin, 1 μg/mL aprotinin, 1 μg/mL pepstatin, 5 μg/mL antipain, 5 μg/mL chymostatin, 2
531 mM $NaVO_3$, 2 mM NaF, 50 μM MG132, 50 μM MG115, 50 μM ALLN). The cleared
532 supernatant was incubated with Protein A beads (Invitrogen, Cat no. 15918-014) with captured
533 anti-GFP (Invitrogen; ab11120) antibody at 4℃ for 2 h. The immune complex was released
534 from the resin by 6×SDS loading buffer. Supernatants were resolved using an 8% SDS-PAGE
535 gel. GFP-tagged TOC1 and PRR5 and HA-tagged PIF4 and PIF5 were detected by western
536 blotting using GFP antibody (Abcam; ab6556) and HA antibody (Roche; 3F10), respectively.
537
538 **Statistical analysis.** Differences between means were statistically analyzed by one-way
539 analysis of variance using Tukey's b *post hoc* multiple comparison test (IBM SPSS Statistics
540 Software) or Student's *t*-test (Excel, Microsoft) as indicated in the figure legends. Statistically

significant differences were defined as those with p values < 0.05. Significance levels are indicated as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Accession numbers

The Arabidopsis Genome Initiative numbers for the genes mentioned in this article are as follows: *TOC1*, *AT5G61380*; *PRR5*, *AT5G24470*; *PRR7*, *AT5G02810*; *PRR9*, *AT2G46790*; *PIF4*, *AT2G43010*; *PIF5*, *AT3G59060*; *YUC8*, *AT4G28720*; *IAA19*, *AT3G15540*; *ATHB2*, *AT4G16780*; *ELF3*, AT2G25930; *ELF4*, AT2G40080; *LUX*, *AT3G46640*. RNA-seq data reported in this study have been deposited in the Gene Expression Omnibus database under accession number GSE99290.

Supplemental Data

Supplemental Figure S1. TOC1 and PRR5 regulate photoperiodic hypocotyl growth independent of LUX.

Supplemental Figure S2. The hypocotyl phenotypes of *prr57, prr59, prr79,* and *prr579* mutants in different photoperiod conditions.

Supplemental Figure S3. Time-course expression pattern of *TOC1*/*PRR5* in short-day or long-day conditions.

Supplemental Figure S4. Time-course expression pattern of *PRRs* and *EC* components in short-day or long-day conditions.

Supplemental Figure S5. Validation of RNA-seq results by reverse transcription quantitative PCR.

Supplemental Figure S6. *PIF4* and *PIF5* were found among the 11 overlapping genes between up-regulated genes in the *toc1 prr5* mutant and co-bound genes by TOC1, PRR5, and PRR7.

Supplemental Figure S7. TOC1 and PRR5 bind the *CCA1* promoter but not the *APX3* promoter.

Supplemental Figure S8. PRR7 and PRR9 directly repress *PIF4* and *PIF5* transcription.

Supplemental Figure S9. The transcriptional pattern of *PIF4* and *PIF5* in *prr* mutants under different photoperiod conditions.

Supplemental Figure S10. Subcellular localization of GFP-TOC1 and GFP-TOC1ΔCCT-NLS proteins.

Supplemental Figure S11. Physical interactions between TOC1, TOC1ΔCCT, TOC1-A562V, and PIF5.

Supplemental Figure S12. The transcriptional inhibition of *PIF4* and *PIF5* by TOC1ΔCCT was significantly attenuated.

Supplemental Table S1. Primers used in this study.

Supplemental Dataset S1. The differentially expressed genes (DEGs) in the *toc1 prr5* double mutant identified by RNA-seq.

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Figure legends

Figure 1. TOC1 and PRR5 coordinate with EC to regulate photoperiodic hypocotyl growth. A, Hypocotyl phenotypes of Col-0, *toc1*, *elf3*, *toc1 elf3*, *prr5*, *prr5 elf3*, *toc1 prr5,* and *toc1 prr5 elf3* seedlings grown under short-day conditions (8L/16D) for 5 days after germination as noted. Scale bar, 5 mm. B**,** Quantitative analysis of the hypocotyl length of the seedlings shown in **A**. Different letters indicate statistically significant differences among averages by Tukey's b test ($p < 0.05$). Data are the means ± SD of more than 15 seedlings. C, Hypocotyl phenotypes of Col-0, *toc1*, *elf3*, *toc1 elf3*, *prr5*, *prr5 elf3*, *toc1 prr5,* and *toc1 prr5 elf3* seedlings grown under

long-day conditions (16L/8D) for 5 days after germination as noted. Scale bar, 5 mm. D, Quantitative analysis of the hypocotyl length of the seedlings shown in (C). Different letters indicate statistically significant differences among averages by Tukey's b test ($p < 0.05$). Data are the means ± SD of more than 15 seedlings. E, Hypocotyl phenotypes of Col-0, *toc1*, *elf3*, *toc1 elf3*, *prr5*, *prr5 elf3*, *toc1 prr5,* and *toc1 prr5 elf3* seedlings grown under continuous white light conditions for 5 days after germination as noted. Scale bar, 5 mm. Seedling images in A, C and E were digitally abstracted and multiple images were made into a composite for comparison. F, Quantitative analysis of the hypocotyl length of the seedlings shown in (E**)**. Different letters indicate statistically significant differences among averages by Tukey's b test ($p < 0.05$). Data are the means ± SD of more than 15 seedlings.

Figure 2. PRR protein expression patterns in differential photoperiod conditions.
A to H, Immunoblots showing TOC1/PRR5/ PRR7/ PRR9 protein abundance in seedlings of *TMG*, *PRR5pro:PRR5-GFP*, *PRR7pro:PRR7-GFP* and *PRR9pro:PRR9-GFP*, respectively, grown in short day or long day conditions for 10 days. Coomassie Brilliant Blue (CBB) staining indicates the protein loading amount. Data are representative of three biological replicates with similar results.

Figure 3. *PIF4* and *PIF5* are potential direct transcriptional targets of TOC1 and PRR5. A, Differentially expressed genes (DEGs) between the *toc1 prr5* mutant and wild-type Col-0 in RNA-seq. The samples were harvested at ZT15 from 10-day-old seedlings grown in 12 h light/12 h dark photocycles. B, Gene ontology (GO) analysis of the overlapping genes between upregulated DEGs in the *toc1 prr5* mutant and the bound genes by TOC1. C, Expression profiles of *PIF4* and *PIF5* in the *toc1 prr5* mutant. Data from RNA-seq. D, Venn diagram showing the number of common genes bound by TOC1, PRR5, and PRR7. E, Protein interaction network analysis of the 90 co-bound genes by TOC1, PRR5, and PRR7 in (D) using the STRING database (http://string-db.org/), showing a major cluster including *PIF4*, *PIF5*, and other known circadian core components. Colored nodes: query proteins and first shell of interactors; white nodes: second shell of interactors; empty nodes: proteins of unknown 3D

structure; filled nodes: some 3D structure is known or predicted. Edges represent protein-protein associations; light blue edges: from curated databases; purple edges: experimentally determined; green edges: gene neighborhood; dark blue: gene co-occurrence; yellow edges: text mining; dark edges: co-expression; light purple edges: protein homology. F, Venn diagram showing the number of overlapping genes among the TOC1, PRR5, and PRR7 co-bound genes, upregulated DEGs in the *toc1 prr5* mutant, and upregulated DEGs in the *lux-6* mutant. G, Heatmap showing 4 common co-targets in upregulated DEGs in *toc1 prr5* and *lux-6* mutants. Scale represents $\log_2$ (fold change).

Figure 4. TOC1 and PRR5 directly bind the *PIF4* and *PIF5* promoters to repress their transcription. A, Schematic diagram of the promoter regions of *PIF4* and *PIF5*. Orange boxes represent the putative G-box elements. G, G1, and G2 represent the respective DNA fragments used for generating EMSA probes and ChIP-qPCR detection. B, EMSA with the CCT domain of TOC1 and PRR5 incubated with a probe designed for the *PIF4-G*, *PIF5-G1*, and *PIF5-G2* regions of the *PIF5* gene as shown in (A), and 100-fold unlabeled competitor (100×). GST alone was used as a negative control. Arrowheads mark the shifted bands. C and D, Time-course ChIP-qPCR assay showing that TOC1 and PRR5 bind to the *PIF4-G* (C) and *PIF5-G2* (D) regions diurnally, which was well associated with their respective protein abundances. Data are the means ± SD. E, Transient transcriptional expression analysis showing that *PIF4* and *PIF5* were repressed by TOC1 and PRR5 in epidermal cells of *N. benthamiana* leaves. *CCA1pro:LUC* was used as a positive control. Data are representative of three biological replicates with similar results. Leaf images were digitally abstracted and multiple images were made into a composite for comparison. F, Quantification of bioluminescence intensity as shown in (E). Data are the means ± SD. The asterisks denote statistically significant differences among means, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ by Student's *t*-test. G and H, Transient transcriptional expression assay in Arabidopsis protoplasts. A schematic diagram of effector and reporter vectors is shown in (G). Respective quantification of relative LUC/GUS activity is shown in (H). The relative LUC/GUS activity in protoplasts co-transformed with GFP and reporter vector was defined as 1. *CCA1pro:LUC* was used as a

positive control, while *35S:GUS* was used as an internal control. Data are the means ± SD. The asterisks in (H) denote statistically significant differences among means, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by Student's *t*-test .

Figure 5. TOC1 and PRR5 coordinate with EC to transmit daylength information for shaping *PIF4* and *PIF5* transcription. A and B, Immunodetection of PIF5 protein levels in *PIF5pro:PIF5-HA* transgenic seedlings. Extracts from seedlings grown in short day (A) and long day (B) conditions for 10 days. CBB staining indicates the protein loading. Data are representative of three biological replicates with similar results. C and D, RT-qPCR analysis showing *PIF5* transcript levels in Col-0, *toc1 prr5*, *elf3,* and *toc1 prr5 elf3* seedlings grown for 10 days in short day (C) or long day (D) conditions. E and F, RT-qPCR analysis showing *PIF4* transcript levels in Col-0, *toc1 prr5*, *elf3,* and *toc1 prr5 elf3* seedlings grown for 10 days in short day (E) and long day (F) conditions. From (C) to (F), data are the means ± SD., white and black rectangles below the graphs represent day and night respectively.

Figure 6. Direct transcriptional inhibition of *PIF4* and *PIF5* by TOC1 is required for its regulation of photoperiodic hypocotyl growth. A, Physical interactions between TOC1, TOC1ΔCCT (1-532aa)-NLS, and PIF4 *in vivo* were detected by co-immunoprecipitation after transient co-expression in *N. benthamiana*. B Hypocotyl phenotypes of *toc1-21*, *GFP-TOC1*/ *toc1-21,* and *GFP-TOC1ΔCCT-NLS* / *toc1-21* transgenic seedlings grown under short day conditions (8L/16D) for 5 days after germination. Seedling images were digitally abstracted and multiple images were made into a composite for comparison. The protein levels of GFP-TOC1 and GFP-TOC1ΔCCT-NLS in these transgenic seedlings were also detected by immunoblot. Representative seedlings were photographed as shown in the left panel, and the hypocotyl lengths of the seedlings shown in the left panel were quantified and are shown in the right panel. Scale bar, 5 mm. Data are the means ± SD of more than 20 seedlings. Different letters indicate statistically significant differences among averages by Tukey's b test ($p < 0.05$). C, RT-qPCR analysis of *PIF4* and *PIF5* expression in *toc1-21*, *GFP-TOC1 toc1-21,* and *GFP-TOC1ΔCCT-NLS toc1-21* transgenic seedlings grown for 10 days in short day conditions

at ZT12. Data are the means ± SD. The asterisks denote statistically significant differences among means, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by Student's *t*-test. D, Physical interaction between TOC1-A562V and PIF4 was detected by co-immunoprecipitation after being transiently co-expressed in leaves of *N. benthamiana*. The immunoprecipitates with human IgG beads were analyzed by immunoblot with anti-PAP or anti-HA antibody as indicated. E, EMSA with CCT and CCT-A562V of TOC1 and GST incubated with a probe designed to the *PIF4-G* and *PIF5-G2* regions, and 100-fold unlabeled competitor (100×). Arrowheads mark the shifted bands. F, Hypocotyl phenotypes of wild type (C24 ecotype) and *toc1-1* grown for 5 days in short day conditions. Representative seedlings were photographed (left panel) and measured (right panel). Data are the means ± SD of more than 20 seedlings. The asterisks denote statistically significant differences among means, ***$p < 0.001$ by Student's *t*-test.

Figure 7. *PIF4* and *PIF5* are epistatic to *TOC1* and *PRR5* for photoperiodic hypocotyl growth. A and B, Hypocotyl phenotypes of Col-0, *toc1*, *pif4*, *toc1 pif4*, *toc1 prr5*, *toc1 prr5 pif4*, *pif4 pif5*, and *toc1 prr5 pif4 pif5* seedlings (5 DAG) grown under short day conditions (8L/16D). C and D, Hypocotyl phenotypes of Col-0, *toc1*, *pif4*, *toc1 pif4*, *toc1 prr5*, *toc1 prr5 pif4*, *pif4 pif5*, and *toc1 prr5 pif4 pif5* seedlings (5 DAG) grown under long day conditions (16L/8D). E and F, Hypocotyl phenotypes of Col-0, *toc1*, *pif4*, *toc1 pif4*, *toc1 prr5*, *toc1 prr5 pif4*, *pif4 pif5*, and *toc1 prr5 pif4 pif5* seedlings (5 DAG) grown under continuous white light conditions. Representative seedlings were photographed as shown in (A), (C) and (E). Seedling images were digitally abstracted and multiple images were made into a composite for comparison. Scale bar, 5 mm. Hypocotyl lengths of the seedlings were measured and quantified as shown in (B), (D) and (F). Different letters indicate statistically significant differences among means by Tukey's b test ($p < 0.05$). Data are the means ± SD of more than 15 seedlings.

Figure 8. A proposed working model for PRR-*PIF4/5* transcriptional module-mediated photoperiodic hypocotyl growth. PSEUDO RESPONSE REGULATORs (PRRs), as core circadian clock components, can directly and sequentially bind the promoters of

PHYTOCHROME INTERACTING FACTOR 4 (*PIF4*) and *PIF5* to repress their transcription in an independent manner with Evenging Complex. Diurnal rhythms of PIF4/5 protein abundance are determined by the coordination of light signaling-mediated protein stability and circadian clock-regulated transcriptional expression. Hence, TOC1 and other PRRs represent a primary molecular node between the circadian clock and photoperiod to control photoperiodic hypocotyl growth.

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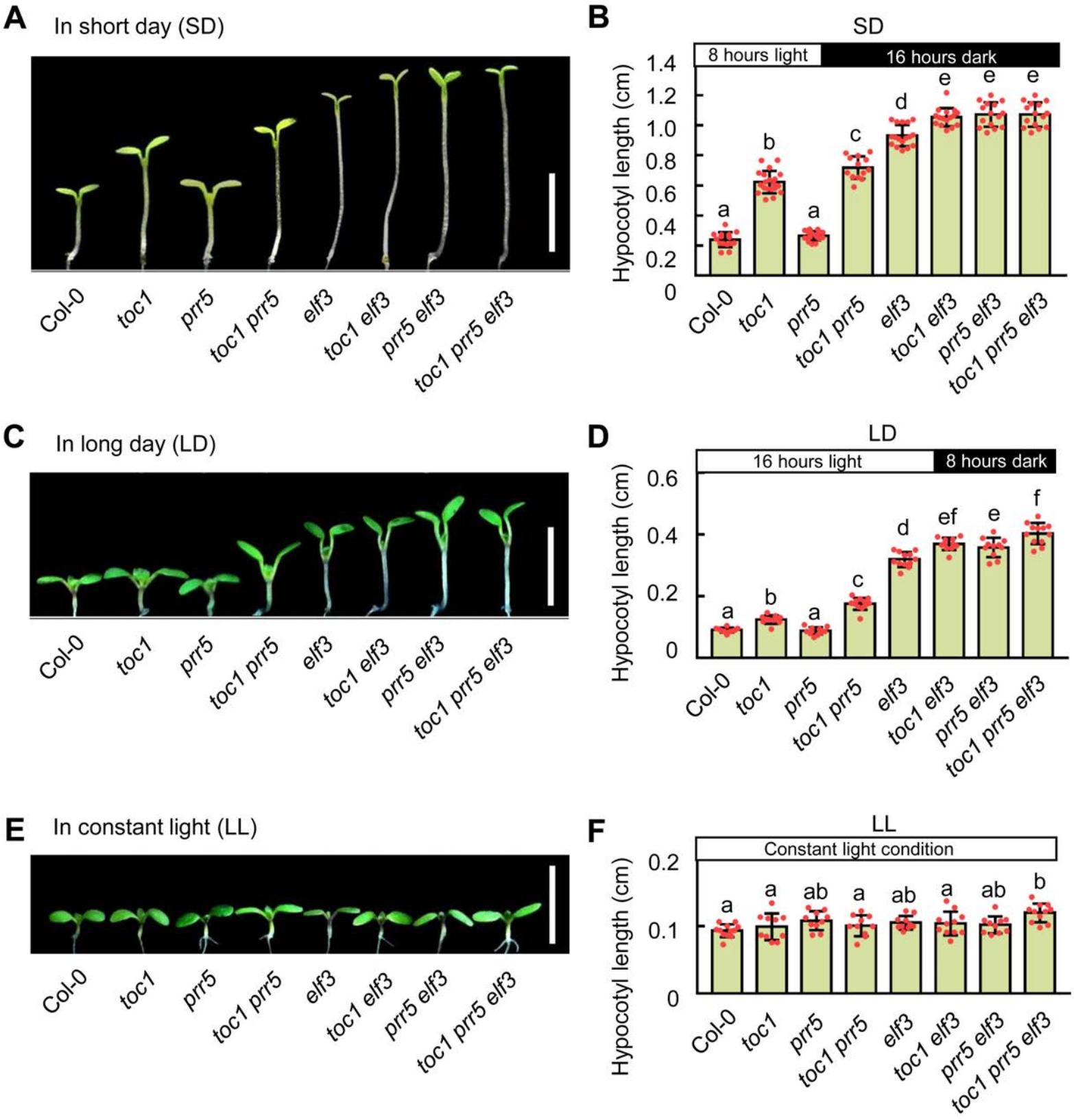

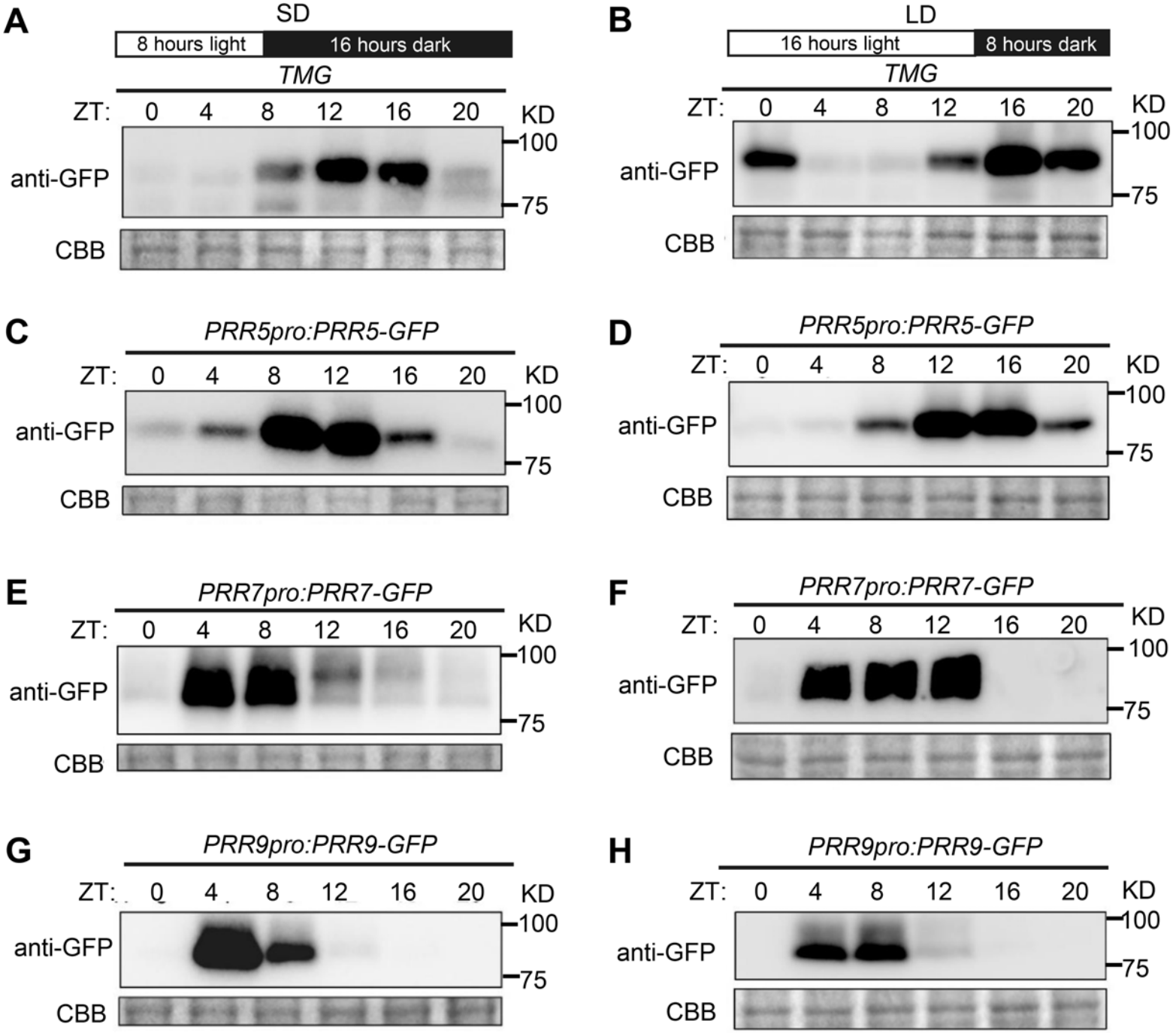

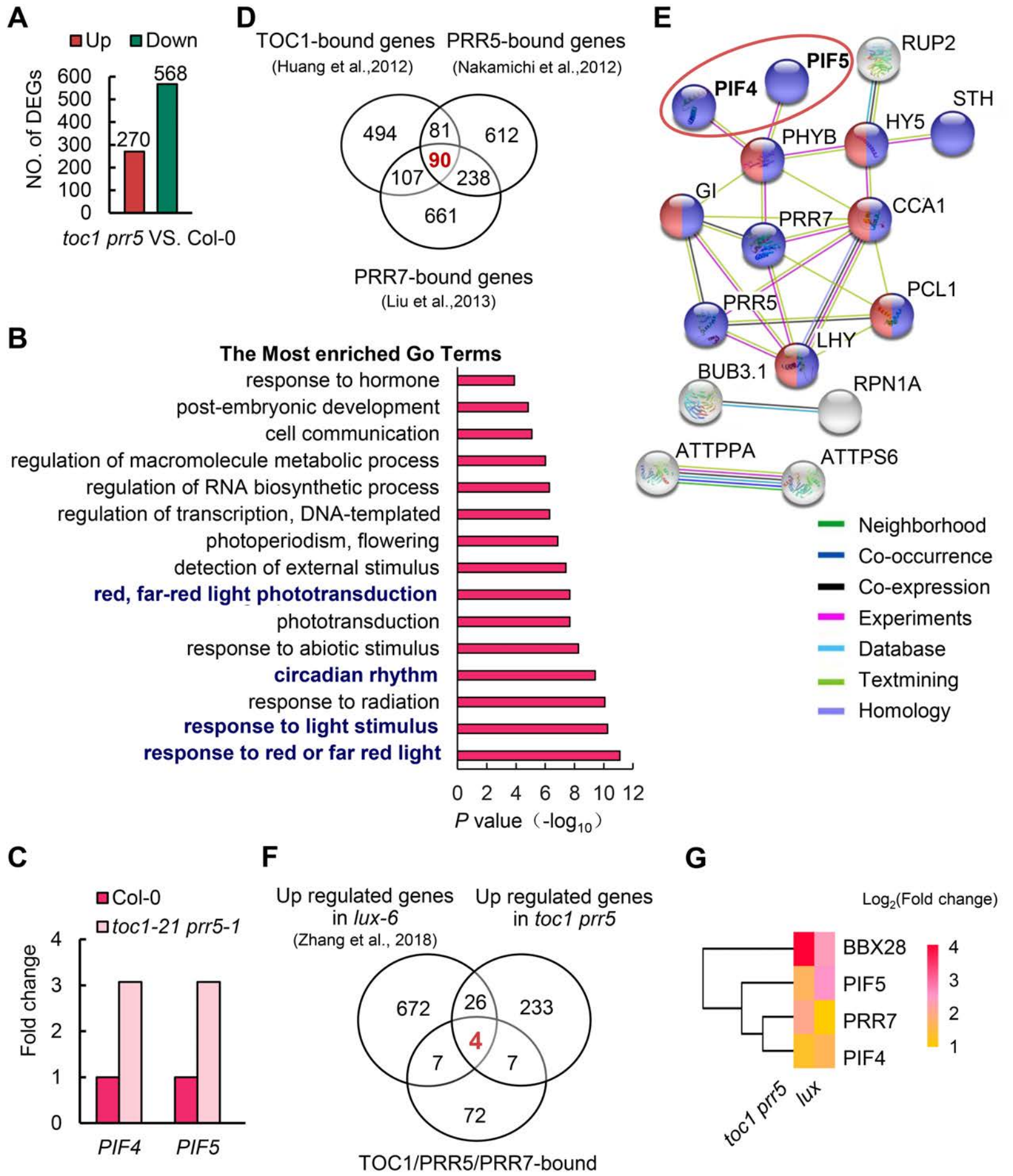

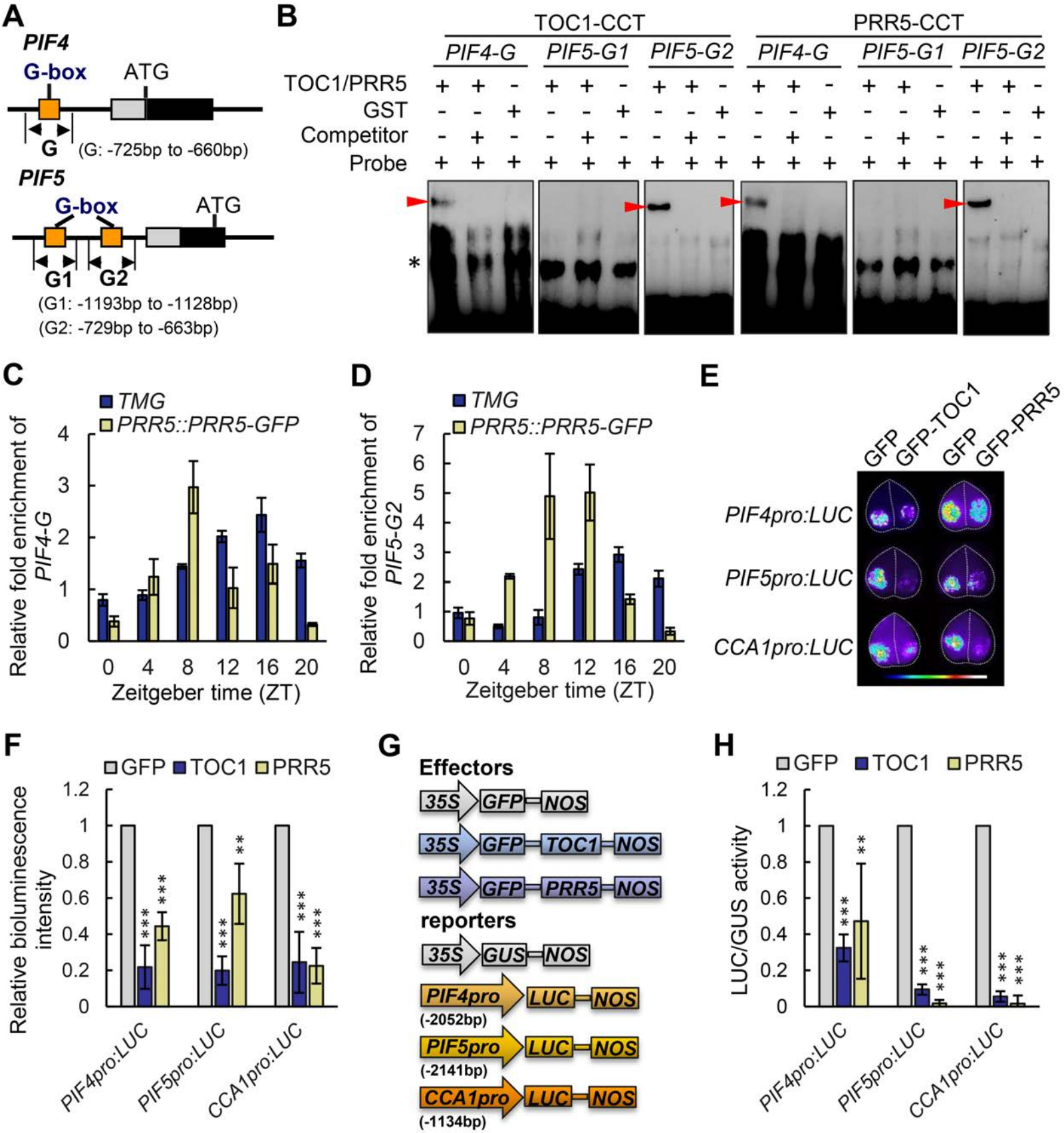

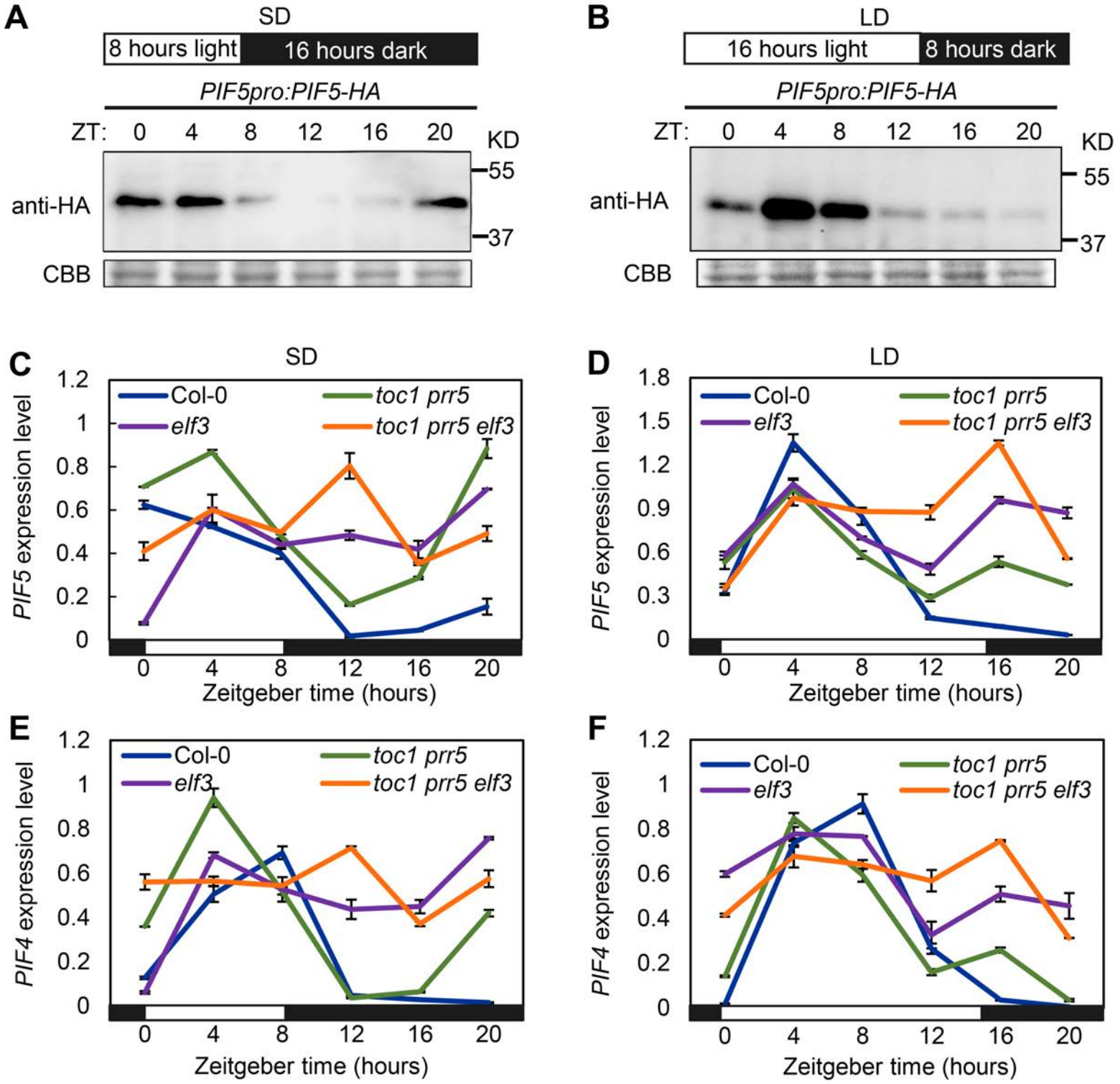

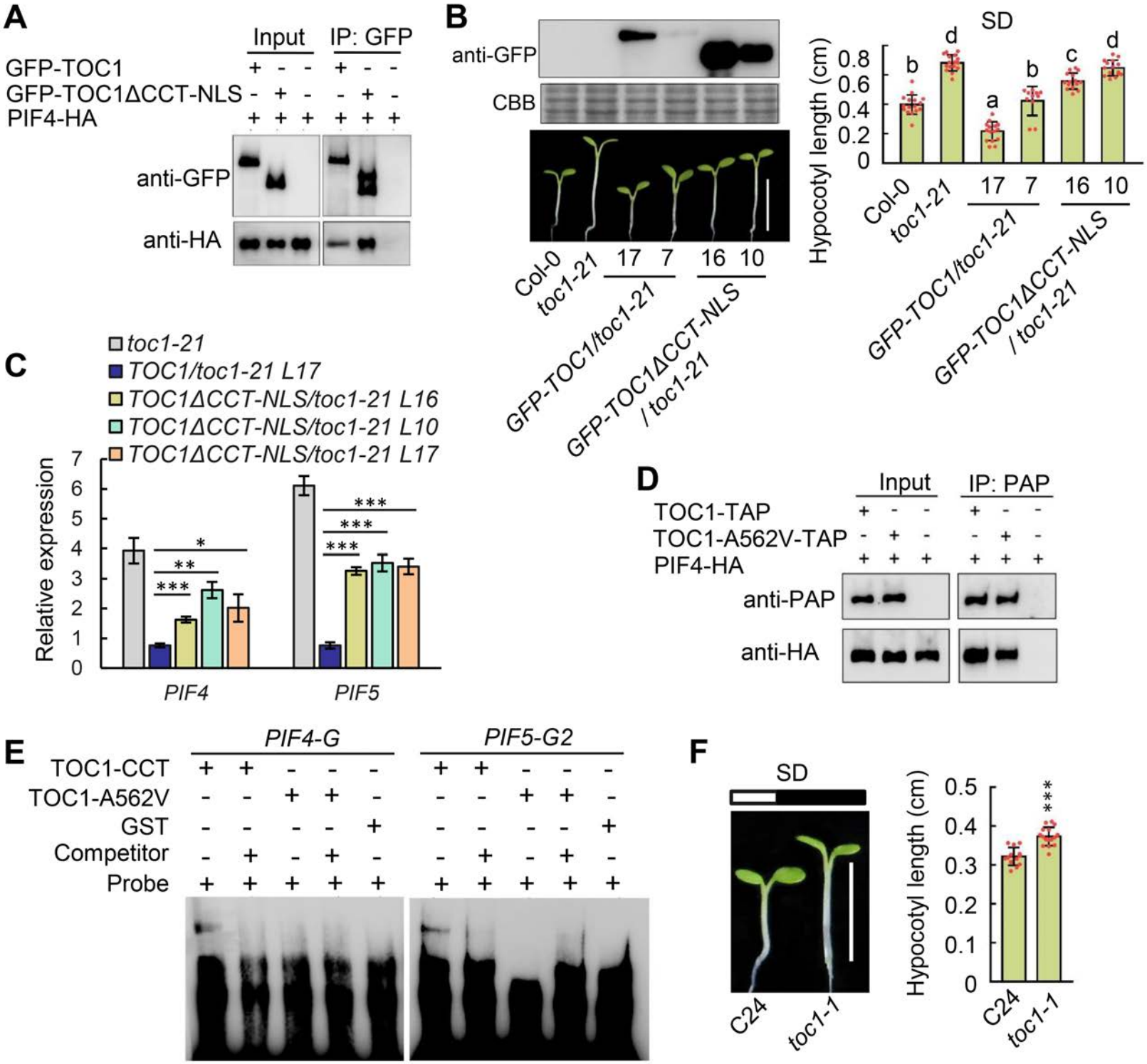

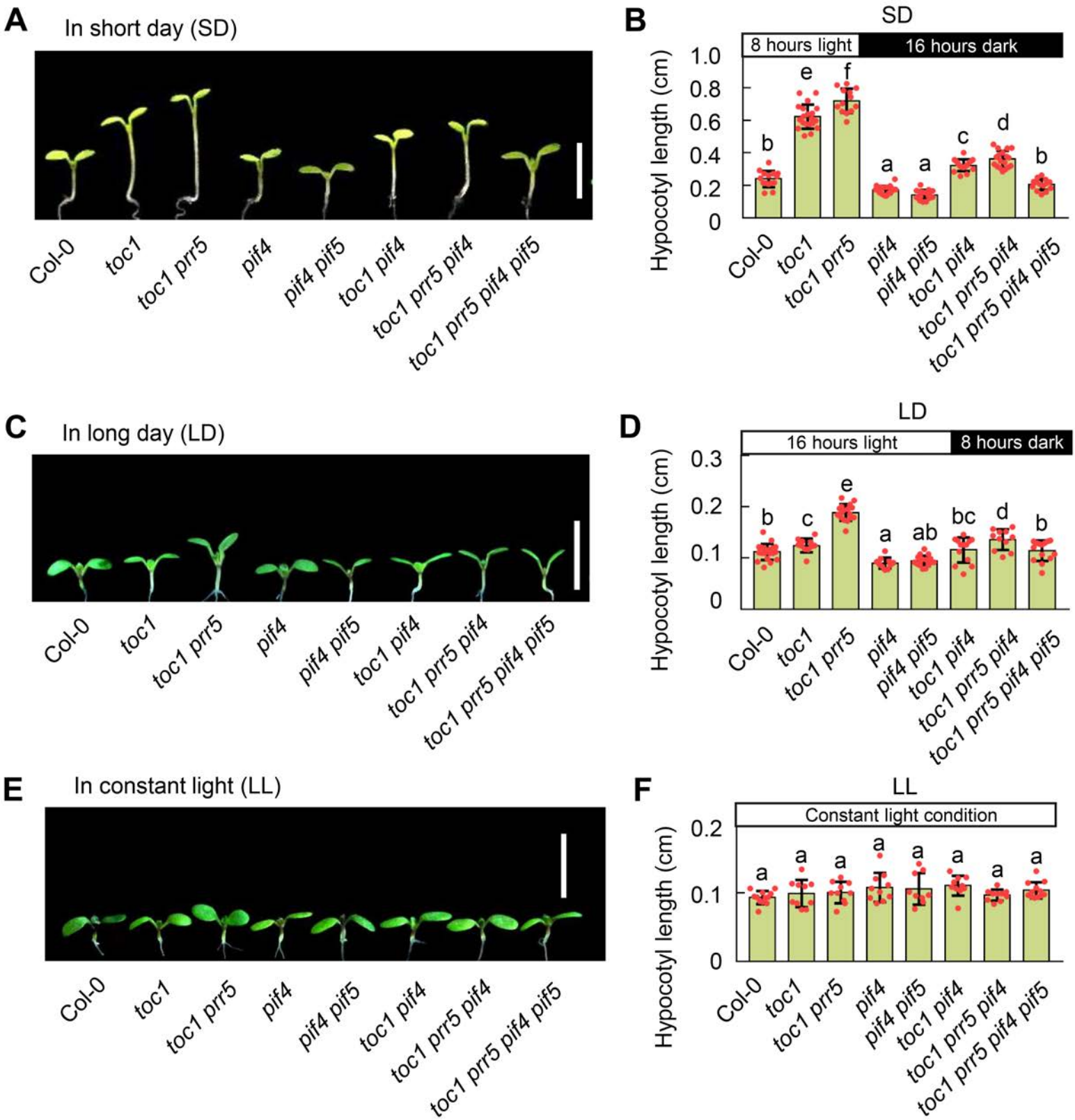

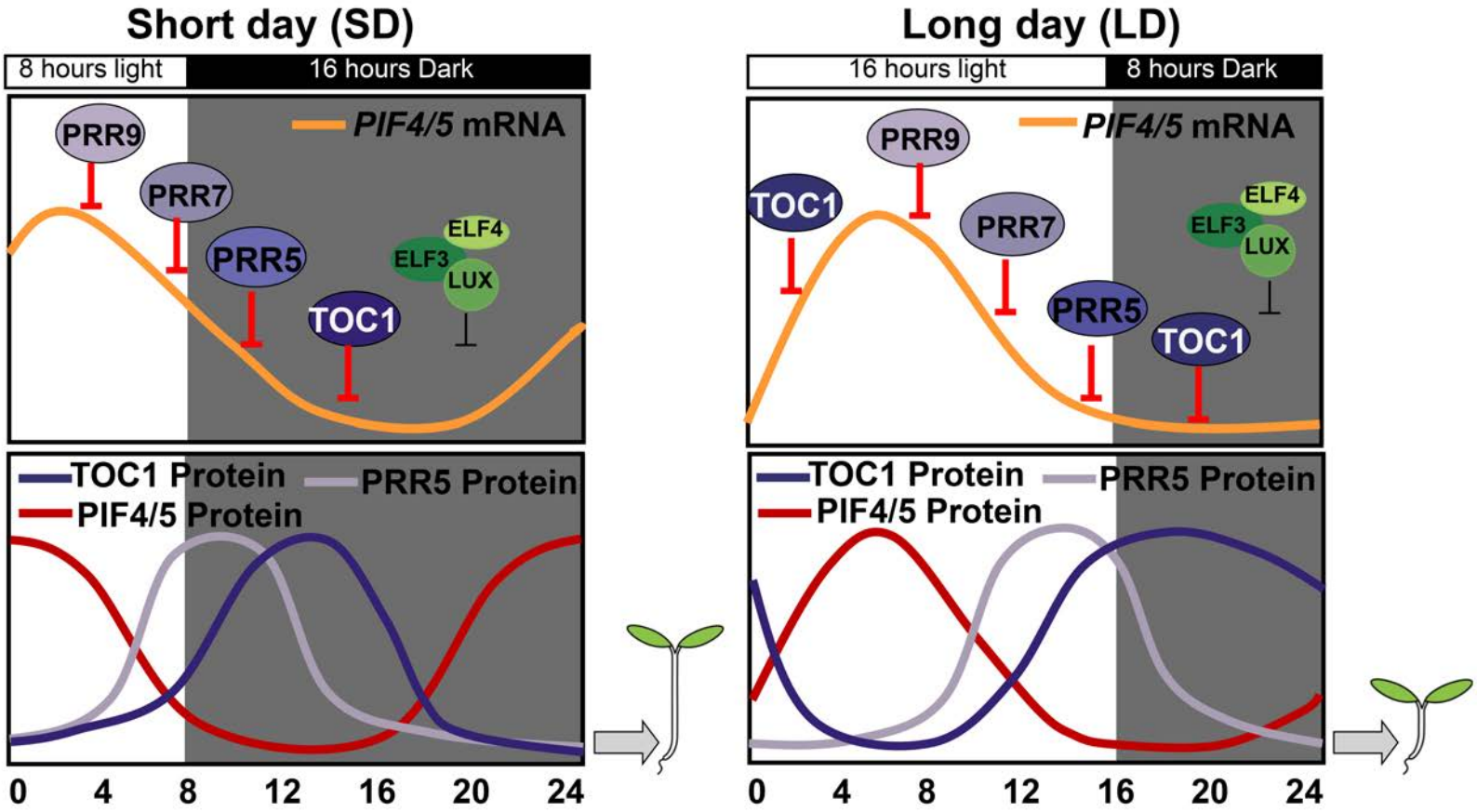

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