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PSEUDO RESPONSE REGULATORs stabilize CONSTANS protein to promote flowering in response to day length

Ryosuke Hayama[†], Liron Sarid-Krebs, René Richter, Virginia Fernández, Seonghoe Jang[‡,§] & George Coupland[*]

Abstract

Seasonal reproduction in many organisms requires detection of day length. This is achieved by integrating information on the light environment with an internal photoperiodic time-keeping mechanism. *Arabidopsis thaliana* promotes flowering in response to long days (LDs), and CONSTANS (CO) transcription factor represents a photoperiodic timer whose stability is higher when plants are exposed to light under LDs. Here, we show that PSEUDO RESPONSE REGULATOR (PRR) proteins directly mediate this stabilization. PRRs interact with and stabilize CO at specific times during the day, thereby mediating its accumulation under LDs. PRR-mediated stabilization increases binding of CO to the promoter of *FLOWERING LOCUS T* (*FT*), leading to enhanced *FT* transcription and early flowering under these conditions. PRRs were previously reported to contribute to timekeeping by regulating *CO* transcription through their roles in the circadian clock. We propose an additional role for PRRs in which they act upon CO protein to promote flowering, directly coupling information on light exposure to the timekeeper and allowing recognition of LDs.

Keywords *Arabidopsis*; circadian clock; CONSTITUTIVE PHOTOMORPHOGENIC 1; photoperiodic flowering; PSEUDO RESPONSE REGULATOR
Subject Categories Plant Biology; Post-translational Modifications, Proteolysis & Proteomics; Transcription
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Introduction

Many organisms recognize seasonal changes in their environment by perceiving day length and utilize this information to control key steps in their life cycle, such as the onset of reproduction or diapause. These responses, referred to as photoperiodism, allow organisms to adapt to high latitude where seasonal climatic fluctuations involving significant temperature changes occur. Photoperiodic responses are generally conferred by a mechanism that allows organisms to measure the length of day or night that fluctuates predictably during the year. This process involves comparison of the light environment against an internal photoperiodic time-keeping mechanism downstream of the circadian clock. In this system, the circadian clock provides an endogenous autonomous rhythm with a period length of ~24 h that regulates the photoperiodic timer.

Day length strongly influences the timing of floral initiation in many plants, allowing them to adapt to higher latitude and enabling successful reproduction. Molecular and genetic studies using the model species *Arabidopsis thaliana*, which flowers specifically in response to photoperiod under long days (LDs) of spring, provided knowledge on the mechanistic framework that couples information on light exposure with the photoperiodic time-keeping mechanism and enables measurement of day length. The *CONSTANS* (*CO*) gene was originally isolated as a photoperiodic floral promoter and defined as the integrator of light and timing information. This is achieved through control of its transcription by the circadian clock and regulation of CO protein stability by light exposure (Andres & Coupland, 2012; Song *et al*, 2014). Accumulation of *CO* transcripts therefore forms a diurnal rhythm for timekeeping under LDs and SDs (Suarez-Lopez *et al*, 2001). Under LDs *CO* transcription is controlled by a clock-controlled blue light photoreceptor FLAVIN-BINDING, KELCH REPEAT, F-BOX1 (FKF1), clock-controlled CYCLING DOF FACTOR (CDFs) transcription factors, and a clock protein GIGANTEA (GI). In this mechanism, a FKF1-GI protein complex temporally promotes transcription of *CO* by binding to and initiating degradation of CDFs, suppressors of *CO* transcription, in a light-dependent manner (Imaizumi *et al*, 2003, 2005; Sawa *et al*, 2007; Fornara *et al*, 2009). Through this regulation, transcripts of *CO* accumulate in the afternoon under LDs when plants are exposed to light. This coincidence between *CO* mRNA and light exposure allows stabilization of translated CO protein at these times, causing

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CO to accumulate under LDs and achieving recognition of LDs (Appendix Fig S1; Valverde *et al*, 2004). CO then directly promotes floral transition through its capacity to activate transcription of the "florigen" gene *FLOWERING LOCUS T* (*FT*) specifically under LDs (Andres & Coupland, 2012; Song *et al*, 2014). By contrast, under SDs, *CO* transcription occurs only in the dark, and under these conditions, CO protein does not accumulate (Valverde *et al*, 2004).

In contrast to current knowledge of the time-keeping mechanism associated with patterns of *CO* mRNA accumulation, how information after light exposure is transferred to CO protein is still unclear. The blue light photoreceptors cryptochrome 1 (cry1) and cryptochrome 2 (cry2) and the far-red photoreceptor phytochrome A (phyA) are required to stabilize CO protein (Valverde *et al*, 2004; Zuo *et al*, 2011). A protein complex of an E3 ubiquitin ligase CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) and SUPPRESSOR OF PHYA1 (SPA1), which are repressors of *A. thaliana* photomorphogenesis, mediates between the photoreceptors and CO protein stabilization (Laubinger *et al*, 2006; Jang *et al*, 2008; Liu *et al*, 2008; Zuo *et al*, 2011; Lau & Deng, 2012; Sarid-Krebs *et al*, 2015). During the night, the COP1/SPA1 protein complex physically interacts with CO in the nuclei to promote its proteasomal degradation, whereas during the day, the phyA and cry photoreceptors promote COP1 accumulation in the cytoplasm thus allowing CO to accumulate in the nucleus (Osterlund & Deng, 1998; Laubinger *et al*, 2006; Jang *et al*, 2008; Liu *et al*, 2008; Zuo *et al*, 2011; Sarid-Krebs *et al*, 2015). Since the COP1/SPA1 complex degrades CO protein during the night, it strongly reduces CO accumulation under SDs (Jang *et al*, 2008; Liu *et al*, 2008). However, in contrast to its clear biological function in the perception of SDs, COP1 also interferes with the recognition of LDs where a residual activity in the light further reduces CO protein levels during the day, perhaps because photoreceptor activity under LDs is insufficient to completely suppress COP1 function (Jang *et al*, 2008). FKF1 might overcome this effect because it has been demonstrated that FKF1 stabilizes CO under LDs (Song *et al*, 2012). However, activity of FKF1 is strictly limited to the afternoon, whereas CO stabilization occurs at other times during the light period, including the morning (Valverde *et al*, 2004; Song *et al*, 2012). Finally, the ubiquitin ligase HOS1 and the APETALA2 (AP2) family protein TARGET OF EAT1 (TOE1), GI, and an E3 ubiquitin ligase ZEITLUPE (ZTL) negatively regulate CO protein abundance, although how their activities are related to light signaling during photoperiodic flowering has not been elucidated (Lazaro *et al*, 2012, 2015; Song *et al*, 2014; Zhang *et al*, 2015). Thus, the mechanisms that ensure CO accumulation in response to light exposure to establish LD recognition and floral transition are not fully understood.

In parallel to the studies of day-length measurement in *A. thaliana*, quantitative trait locus (QTL) analyses of flowering time identified *PSEUDO RESPONSE REGULATOR* (*PRR*) genes in a wide range of crop species. *Ppd-H1*, *Ppd-1*, *BOLTING TIME CONTROL 1 (BvBTC1)*, *PRR37,* and *SbPRR37* encode PRR proteins in barley, wheat, beet, rice, and sorghum, respectively, and allelic variation at these genes confers natural diversity in flowering time (Turner *et al*, 2005; Pin *et al*, 2009; Murphy *et al*, 2011; Campoli *et al*, 2012; Shaw *et al*, 2012; Koo *et al*, 2013). In *A. thaliana,* PRR proteins are encoded by a family of five genes and are defined as central components of the circadian clock (*TIMING OF CAB EXPRESSION 1* (*TOC1*), *PRR3*, *PRR5*, *PRR7,* and *PRR9*) (Strayer *et al*, 2000; Ito *et al*, 2003; Yamamoto *et al*, 2003; Murakami *et al*, 2004; Para *et al*, 2007). The abundance of their transcripts and proteins exhibit circadian rhythms, and under diurnal conditions, they peak in expression sequentially at 2–3 h intervals during the light period in the order *PRR9*, *PRR7*, *PRR5*, *PRR3*, and *TOC1* (Matsushika *et al*, 2000; Fujiwara *et al*, 2008). PRR9, PRR7, PRR5, and TOC1 proteins are degraded during the night, so they mainly accumulate during the day when they repress transcription of genes encoding other clock components such as LATE ELONGATED HYPOCOTYL (LHY) and CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) (Mas *et al*, 2003b; Farré & Kay, 2007; Ito *et al*, 2007; Kiba *et al*, 2007; Nakamichi *et al*, 2010; Huang *et al*, 2012). Mutations in the *PRR* genes also delay flowering under LDs (Nakamichi *et al*, 2005; Ito *et al*, 2008), but double or triple mutants exhibit much stronger phenotypes suggesting functional redundancy between the genes (Nakamichi *et al*, 2005, p. 549; Ito *et al*, 2008). Despite the significance of the PRRs in flowering, how they contribute to this process remains unclear. In *A. thaliana,* they appear to influence the time-keeping mechanism associated with *CO* transcription in the photoperiodic flowering pathway indirectly through their role in the circadian clock (Nakamichi *et al*, 2007; Ito *et al*, 2008). On the other hand, in crops such as barley and rice, their effects on *CO* transcription are relatively weak, whereas they strongly affect *FT* transcription (Turner *et al*, 2005; Campoli *et al*, 2012; Koo *et al*, 2013). Therefore, these PRRs are likely to have a more direct effect on the regulation of the photoperiodic flowering pathway.

Here, we identify novel mechanisms contributing to day-length measurement and define unexpectedly direct roles for PRRs in photoperiodic flowering. We show that PRRs convey information on light exposure to the photoperiodic time-keeping mechanism by interacting with and stabilizing CO during the day, thereby allowing CO to accumulate at higher levels under LDs. In *A. thaliana*, diversity in temporal accumulation patterns among PRRs throughout the day contributes to detection of long-light periods and to CO accumulation in the morning and the evening specifically under LDs. Besides the function of PRRs in controlling the activity of the photoperiodic time-keeping mechanism associated with *CO* transcription through their role the circadian clock, we find unexpectedly that PRRs also transfer information on light exposure and day length to CO at the post-translational level. This level of regulation allows CO to optimally respond to LDs and accelerates the floral transition under these conditions.

Results

PRRs induce *FT* transcription independently of *CO* transcription

In *A. thaliana toc1 prr5*, *prr5 prr7,* and *prr7 prr9* double mutants, the levels of *CO* mRNA are slightly reduced, contributing to late flowering (Nakamichi *et al*, 2007; Ito *et al*, 2008). However, in these mutants, transcript levels of *FLOWERING LOCUS T* (*FT*), which is a direct target of CO and encodes a florigen protein (Tiwari *et al*, 2010; Andres & Coupland, 2012; Song *et al*, 2012), are drastically reduced. We confirmed the effects of *prr5 prr7* and *prr7 prr9* mutations on *CO* and *FT* mRNAs under LD. *CO* mRNA in these mutants was lower than in wild-type (WT) plants but still accumulated at some times, particularly 12 and 16 h after dawn, whereas *FT* mRNA

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was strongly reduced throughout the day (Fig EV1A and B). The effects of overexpression of *PRR5* and *PRR9* on *CO* and *FT* mRNA levels under LD were then tested. The abundance of *CO* mRNA was slightly lower in the overexpression plants compared to WT, especially at 12 and 16 h after dawn, whereas *FT* mRNA levels were inversely related to *CO* mRNA and elevated at these times (Fig 1A and B). These results are consistent with a hypothesis that PRRs upregulate *FT* mRNA level independently from affecting *CO* mRNA levels.

To further analyze the role of PRRs in *FT* transcription, the effects of *prr* mutations on *FT* expression were tested in a transgenic line expressing *CO* from the *SUC2* promoter (An *et al*, 2004), thereby uncoupling *CO* transcription from impaired clock function in *prr* mutants. The *SUC2* promoter expresses CO specifically in the phloem companion cells where it acts in WT plants to promote *FT* transcription (An *et al*, 2004). In these plants, *FT* expression was highly induced compared with WT due to increased accumulation of *CO* mRNA specifically in the phloem cells (Fig 2A and B). The *prr* mutations were introduced singly and in combination into *SUC2*::*CO* plants. Single *prr5*, *prr7*, *prr9*, or *toc1* mutations in *SUC2*::*CO* background did not dramatically affect *CO* or *FT* expression (Fig EV1C and D). By contrast, combining *prr* mutations in *SUC2*::*CO* reduced *FT* mRNA levels without significantly affecting *CO* mRNA (Fig 2C and D). In *toc1 prr5 SUC2*::*CO*, the levels of *FT* mRNA were slightly reduced compared to *SUC2*::*CO*. In *toc1 prr5 prr7 SUC2*::*CO* plants, *FT* mRNA was further reduced, and this effect was even more enhanced in *toc1 prr5 prr7 prr9 SUC2*::*CO* plants (Fig 2C and D). The flowering times of these lines were also measured, and the *toc1 prr5 prr7 SUC2*::*CO* as well as the *toc1 prr5 prr7 prr9 SUC2*::*CO* line exhibited late-flowering phenotypes compared to *SUC2*::*CO*, consistent with their reduced *FT* mRNA levels (Figs 2E and EV1E). Although *FT* mRNA levels were reduced in *toc1 prr5 SUC2*::*CO* plants compared to *SUC2*::*CO*, the mutant line was only mildly later flowering, presumably because *FT* levels are still so high in *toc1 prr5 SUC2*::*CO* that their effect on flowering is close to saturation. These results demonstrate that PRR proteins promote *FT* transcription independently from *CO* transcription.

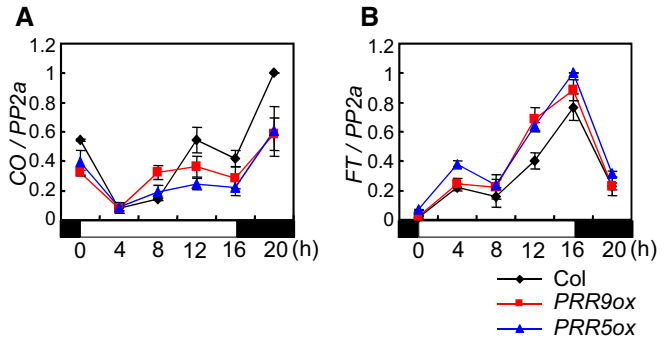

Figure 1. *PRRs* induce *FT* transcription independently from regulating *CO* transcription.

A, B Expression pattern of *CO* (A) and *FT* (B) mRNA in *PRR5*- or *PRR9*-overexpressors under LD. The values of *CO* and *FT* mRNA levels were normalized to those of *PP2a*. For all data, error bars indicate standard error within two biological replicates and in each of these two technical replicates were performed.

PRRs stabilize CO protein under LD

Based on the observation that *prr* mutations can reduce *FT* transcript levels independently from *CO* mRNA, we hypothesized that

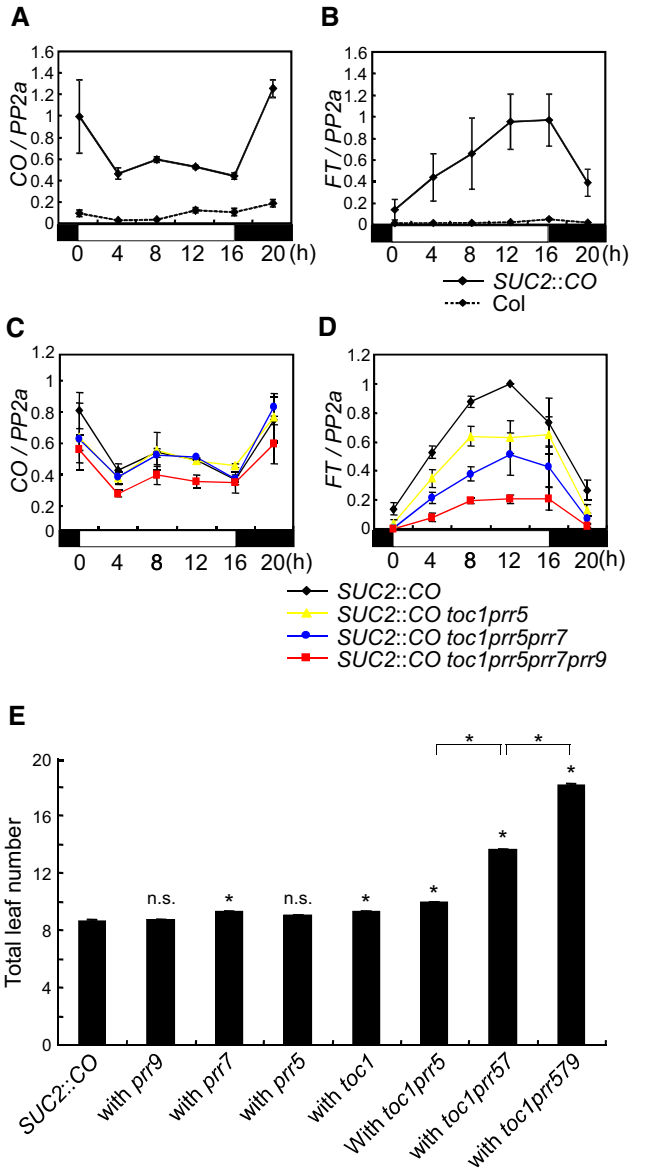

Figure 2. *PRR* genes contribute to *SUC2*::*CO*-mediated *FT* transcription.

A, B Comparison of *CO* (A) and *FT* (B) mRNA levels between WT and *SUC2*::*CO*.

C, D Effect of double, triple, and quadruple *prr* mutations on *CO* (C) and *FT* (D) mRNA in *SUC2*::*CO* background under LDs. The values of *CO* and *FT* mRNA levels were normalized to those of *PP2a*.

E Effect of *prr* mutations on flowering time in *SUC2*::*CO* background under LDs. For each line, 16 plants were used for flowering-time measurement. Error bars indicate standard error. Statistical significance between *SUC2*::*CO* and each *prr* mutant, and among multiple *prr* mutants was calculated using Student's *t*-test; $*P < 0.01$; n.s. $P > 0.01$.

Data information: For data in (A–D), error bars indicate standard error within two biological replicates and for each of these two technical replicates were performed.

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the *prr* mutations act in part at the post-transcriptional level to reduce CO protein abundance. To test this idea, the *prr* mutations were introduced into *SUC2*::*HA:CO*, which allowed us to monitor HA:CO protein abundance in the *prr* mutants. In *SUC2*::*HA:CO* under LD, HA:CO protein accumulated during the day, but its levels were rapidly decreased in the night, although *CO* mRNA abundance remained high at this time (Fig 3A, B and D). This result is consistent with previous data demonstrating that in 35S::*CO* plants, where *CO* mRNA is constantly expressed, CO protein accumulates in the day and rapidly disappears in the night, due to light-mediated stabilization of CO protein (Valverde *et al*, 2004; Song *et al*, 2012). During the light period under LD, HA:CO levels in *toc1 prr5 SUC2*::*HA:CO* were slightly reduced compared to *SUC2*::*HA:CO* and a further reduction of HA:CO protein was observed in *toc1 prr5 prr7 SUC2*::*HA:CO* and even more dramatically in *toc1 prr5 prr7 prr9 SUC2*::*HA:CO* mutants (Fig 3A and B). In these lines, *FT* mRNA levels were reduced and late flowering was also observed, consistent with the reduced HA:CO protein levels (Figs 3C and EV2F). *CO* mRNA levels were not significantly affected in these lines (Fig 3D). Taken together, these data indicate that the PRRs act redundantly to enhance CO protein accumulation under LDs.

When *CO* mRNA is expressed constitutively from the *35S* promoter under LDs, CO protein peaks in abundance early in the morning and again in the afternoon (Valverde *et al*, 2004; Song *et al*, 2012). We tested this pattern in *pCO*::*HA:CO* lines in which *HA:CO* is expressed from the endogenous *CO* promoter. As in the *35S* lines, the levels of HA:CO protein increased early in the morning and in the afternoon, and these peaks appeared more strongly under LD than SD (Figs 3E, F and H, and EV2B–D). The diurnal accumulation of *FT* mRNA was also similar to that of HA:CO protein (Figs 3G and EV2E), suggesting that both the morning and the evening peak of CO contribute to LD-induction of flowering. In a *co* mutant induction of *FT* in the morning was impaired, indicating that *FT* transcription in the morning depends on *CO* activity (Fig EV2E; Kim *et al*, 2005). Next, we analyzed the temporal HA:CO protein accumulation in single and higher order *prr* mutants at different times during the day and compared those to the WT. In agreement with previous reports on PRR9 function in the morning, HA:CO accumulation was specifically reduced in the morning in *prr9 SUC2*::*HA:CO* plants (Figs 3I–L and EV3A), whereas the *toc1 prr5 prr7* mutations strongly reduced the evening peak under LD (Fig 3I–L; Fujiwara *et al*, 2008). These data indicate that diversity in the timing of expression and activity of PRRs cause CO to accumulate at specific times during the day to generate the typical LD-specific accumulation pattern. Previously, FKF1 was reported to stabilize CO protein specifically in the evening in *35S*::*CO* plants (Song *et al*, 2012). However, in contrast to the dramatic reduction of HA:CO levels in *toc1 prr5 prr7 prr9 SUC2*::*HA:CO*, the *fkf1* mutation did not strongly affect HA:CO levels in the same *SUC2*::*HA:CO* line (Fig EV3C–E). This result suggests that PRRs are more heavily involved than FKF1 in increasing CO protein levels when it is expressed from the *SUC2* promoter in the phloem companion cells, the tissue in which CO promotes floral induction.

PRRs contribute to light-mediated accumulation of CO

PRR proteins accumulate during the day and are degraded during the night (Mas *et al*, 2003b; Farré & Kay, 2007; Ito *et al*, 2007; Kiba *et al*, 2007; Fujiwara *et al*, 2008), so they could directly contribute to CO protein stabilization in light. CO is also known to be stabilized in plants exposed to blue light (BL) and far-red light (FR) (Valverde *et al*, 2004). To understand whether CO protein abundance under different light regimes is dependent on PRR function, HA:CO protein accumulation was followed under BL and FR in *SUC2*::*HA:CO* plants and after introduction of *prr* mutations. In *SUC2*::*HA:CO* plants, the levels of HA:CO protein were low in the dark, but increased strongly in BL or FR independently of *CO* mRNA abundance (Fig 4A and B). The *toc1 prr5 prr7 SUC2*::*HA:CO* and *toc1 prr5 prr7 prr9 SUC2*::*HA:CO* lines showed strongly reduced HA:CO protein levels in both BL and FR when compared to *SUC2*::*HA:CO* (Fig 4A and B). Moreover, *FT* mRNA levels under both conditions correlated with the abundance of HA:CO protein, but not with *CO* mRNA (Fig 4C and D). Together with the observations that PRR proteins accumulate during the day, these results support the hypothesis that the PRR proteins contribute to light-mediated accumulation of CO.

In *A. thaliana* light-signaling pathways, COP1 acts as a negative regulator to suppress photomorphogenesis by targeting proteins such as the transcription factor ELONGATED HYPOCOTYL 5 (HY5) for proteasomal degradation during the dark (Osterlund *et al*, 2000). In the light, phys and crys suppress COP1 function, allowing HY5 to accumulate and promote photomorphogenesis (Osterlund *et al*, 2000). The photoreceptor-COP1 module also controls CO protein abundance, where phyA, cry1, and cry2 suppress the activity of COP1 to degrade CO thereby allowing CO to accumulate during the day (Zuo *et al*, 2011; Sarid-Krebs *et al*, 2015). As PRRs stabilize CO protein during the day, we tested whether they might also act by suppressing COP1 activity at this time. HA:CO protein abundance was therefore compared in the *toc1 prr5 prr7 prr9 SUC2*::*HA:CO* line and a *cop1 toc1 prr5 prr7 prr9 SUC2*::*HA:CO* line that we constructed. In agreement with our previous observation, HA:CO protein levels were strongly reduced in the *toc1 prr5 prr7 prr9 SUC2*::*HA:CO* line but were increased by introduction of the *cop1* mutation in the *cop1 toc1 prr5 prr7 prr9 SUC2*::*HA:CO* line (Fig 4E and F). Interestingly, the quintuple mutant *cop1 toc1 prr5 prr7 prr9 SUC2*::*HA:CO* contained similar amounts of HA:CO protein to *cop1 SUC2*::*HA:CO* controls (Fig 4E and F), demonstrating that the *cop1* mutation is epistatic to the *prr* mutations with respect to CO protein abundance. Also, the higher abundance of HA:CO protein in the presence of the *cop1* mutation could not be explained by increased levels of *CO* mRNA (Fig 4G). These results demonstrate that reduced CO abundance in *prr* mutants requires COP1 activity, and suggest that PRRs stabilize CO by suppressing *COP1*-mediated degradation of CO in the light. We also tested whether PRRs contribute to HA:CO accumulation by suppressing its proteasome-mediated degradation. *SUC2*::*HA:CO* and *toc1 prr5 prr7 prr9 SUC2*::*HA:CO* plants were treated with the proteasome inhibitor MG132, and the increase in HA:CO accumulation after MG132 treatment was compared between genotypes. In the *toc1 prr5 prr7 prr9 SUC2*::*HA:CO* line, HA:CO levels increased to a greater extent after MG132 treatment than in *SUC2*::*HA:CO* plants (Fig EV2A), supporting the idea that PRRs suppress proteasomal degradation of CO.

The abundance of HY5 protein was then compared in *SUC2*::*HA:CO* and *toc1 prr5 prr7 prr9 SUC2*::*HA:CO* plants, because if the PRRs generally suppress COP1 activity then in the *prr* quadruple mutant

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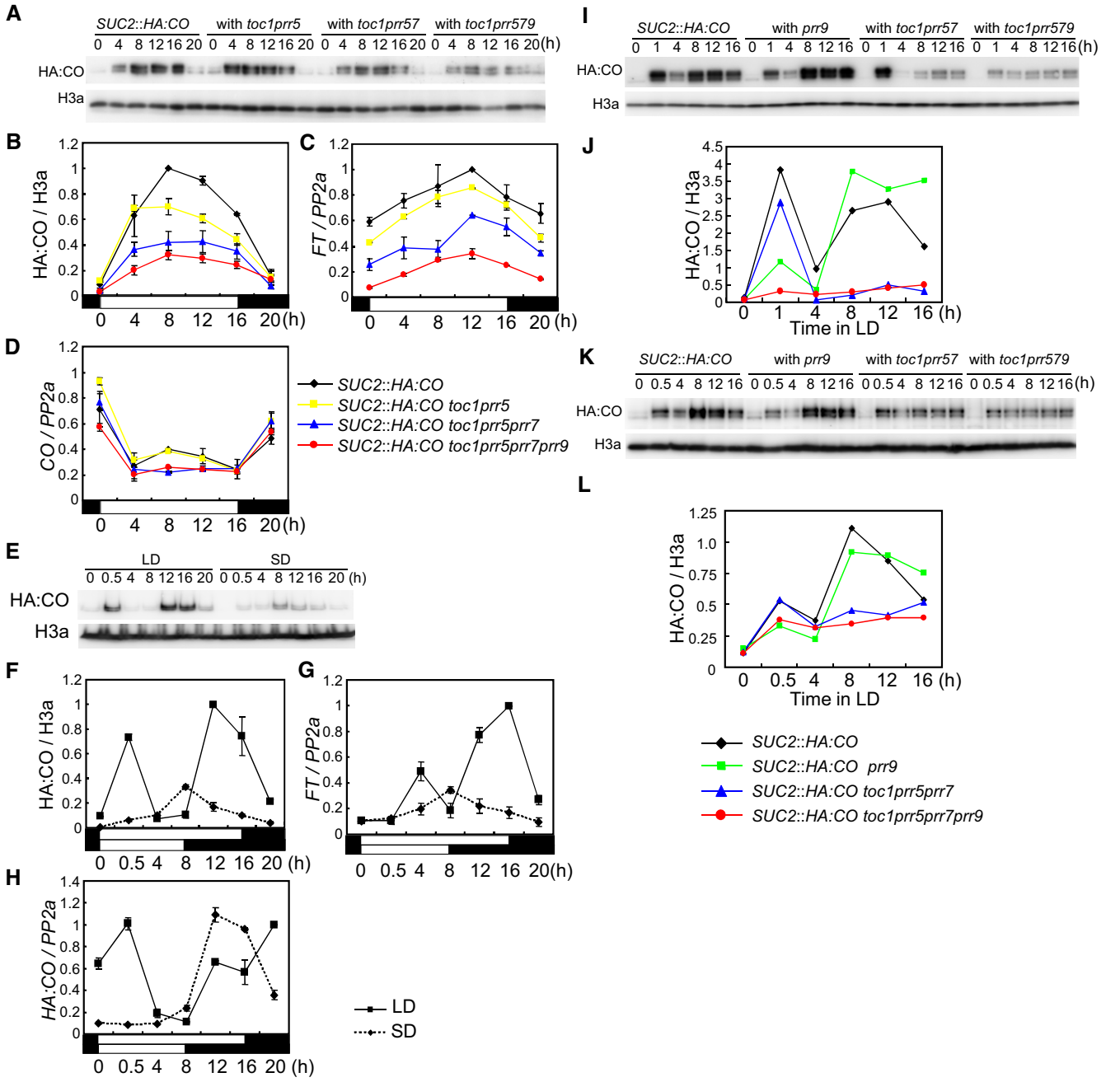

Figure 3. *PRR* genes contribute to CO protein accumulation under LD.

A, B Effect of double, triple, and quadruple *prr* mutations on HA:CO protein accumulation in *SUC2::HA:CO* under LD.

C, D *FT* (C) and *CO* (D) mRNA expression under the same conditions as in (A, B).

E, F Expression pattern of HA:CO protein in *pCO::HA:CO* under LD and SD.

G, H *FT* (G) and *HA:CO* (H) mRNA expression in *pCO::HA:CO* under LD and SD.

I–L Effect of *prr9* and *toc1 prr5 prr7* mutations on HA:CO protein accumulation in *SUC2::HA:CO* background under LD. Samples collected early in the day were harvested at *Zeitgeber* time (ZT) 1 for (I and J) and at ZT 0.5 for (K and L).

Data information: The values of HA:CO levels were normalized to those of histone 3a. The values of *CO* and *FT* mRNA levels were normalized to those of *PP2a*. For all data except for (B), error bars indicate standard error within two biological replicates. For (B), error bars indicate standard error within three biological replicates. For the RNA analyses, two technical replicates were performed for each biological replicate.

background the level of HY5 should be reduced due to increased COP1 function. Consistent with this hypothesis, impairing the function of several *PRRs* causes a long-hypocotyl phenotype in *A. thaliana* similar to that of *hy5* mutants (Fig EV4A; Yamashino *et al*, 2008). Unexpectedly, however, the levels of HY5 protein were not reduced in the light in the *toc1 prr5 prr7 prr9 SUC2::HA:CO*

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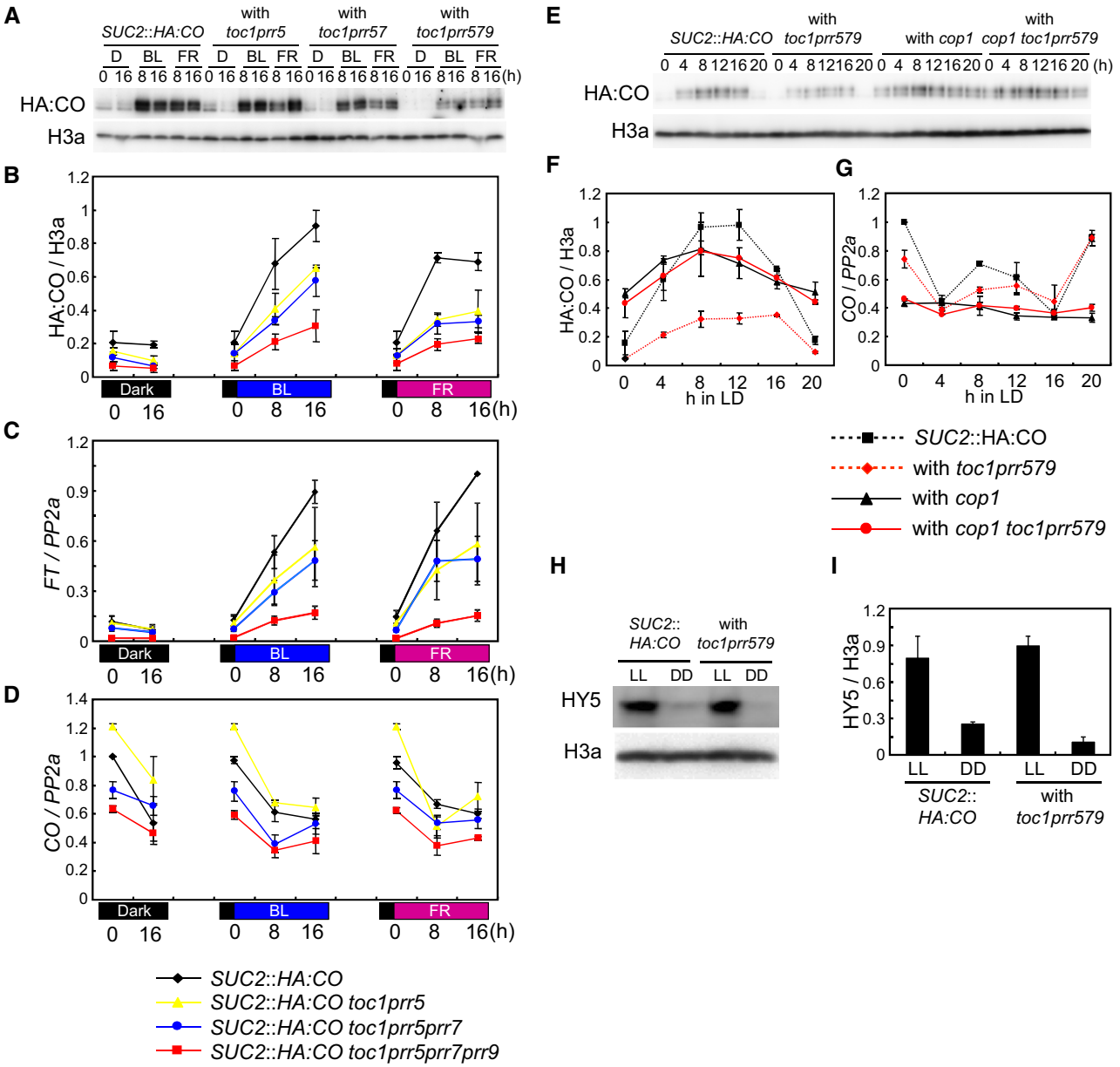

Figure 4. *PRR* genes are required for BL- and FR-mediated CO protein accumulation.

A, B Effect of double, triple, and quadruple *prr* mutations on HA:CO protein accumulation in blue light (BL) and far-red light (FR). Plants were grown under LDs and then transferred to darkness for 24 h. The plants were then transferred to continuous BL or FR at ZT 0. A population of plants was kept in darkness (D) as control. HA:CO protein was analyzed in each genotype at the illustrated times under BL, FR, or control D treatment.

C *FT* mRNA accumulation under the same conditions as in (A, B).

D *CO* mRNA accumulation under the same conditions as in (A, B).

E–G *PRRs* suppress ability of COP1 to degrade CO. (E, F) Effect of quadruple *prr*, *cop1*, and quintuple *prr/cop1* mutations on CO protein abundance in *SUC2::HA:CO* background through a time course under LDs. (G) *CO* mRNA levels in genotypes used in (F).

H, I Abundance of HY5 protein in *prr* quadruple mutant in continuous light (LL) and continuous dark (DD). Plants were grown under LDs for 7 days, transferred to LL or DD at ZT 0 and kept for 40 h before harvesting.

Data information: The values of HA:CO and HY5 levels were normalized to those of histone 3a. Student's *t*-test revealed no significant difference between genotypes. The values of *CO* and *FT* mRNA levels were normalized by those of *PP2a*. For all data except for (B), error bars indicate standard error within two biological replicates. For (B), error bars indicate standard error within three biological replicates. For the RNA analyses, two technical replicates were performed in each biological replicate.

quadruple mutant (Fig 4H and I), although under the same conditions the levels of HA:CO protein were decreased. This result suggests that *PRRs* do not regulate all of the activities of COP1, but that they interfere more specifically with its function to target CO for proteasomal degradation. Furthermore, we also found that the long-hypocotyl phenotype of *toc1 prr5 prr7 prr9* is observed under

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LDs and SDs but not in continuous light (Fig EV4A), although COP1 overexpression was reported to cause a long hypocotyl even under this condition (Torii *et al*, 1998). These data also suggest that the reduction of CO protein and the long-hypocotyl phenotype in the *prr* quadruple mutant are not caused by a general increase in *COP1* activity. The long hypocotyl of the *prr* quadruple mutant may rather be due to impaired circadian-clock function, causing hyper-accumulation of transcripts of a clock-controlled gene, such as *PHYTOCHROME INTERACTING FACTOR 4* (*PIF4*), whose mRNA was increased in abundance in this background and encodes a transcription factor involved in promotion of hypocotyl growth in shade and dark (Fig EV4B; Franklin, 2008; Nakamichi *et al*, 2009). Accumulation of PIF4 protein requires shade or darkness (Nozue *et al*, 2007; Leivar *et al*, 2008; Lorrain *et al*, 2008), consistent with our data that the long-hypocotyl phenotype in the *prr* mutant was observed only under LD and SD conditions, which include periods of darkness.

PRRs stabilize CO independently from altering expression of other clock-controlled genes

Circadian-clock function is strongly impaired in the triple mutant *prr5 prr7 prr9* (Nakamichi *et al*, 2005), suggesting that the observed effect of the quadruple *toc1 prr5 prr7 prr9* mutant on HA:CO protein level could be due to altered expression of other clock-regulated genes that affect CO accumulation. For example, the mRNA level of the clock-controlled gene *FKF1* was reduced in the quadruple mutant (Appendix Fig S2C). However, the abundance of HA:CO protein in *fkf1 SUC2::HA:CO* plants was similar to *SUC2::HA:CO* and clearly more than *toc1 prr5 prr7 prr9 SUC2::HA:CO* (Fig EV3C–E), indicating that the reduced HA:CO protein level in *toc1 prr5 prr7 prr9 SUC2::HA:CO* plants is not explained by reduced *FKF1* activity. Moreover, in contrast to the late-flowering phenotype of *toc1 prr5 prr7 prr9 SUC2::HA:CO* plants, the *fkf1 SUC2::HA:CO* line flowered at the same time as *SUC2::HA:CO* (Appendix Fig S2F). Also, although *fkf1* mutation was reported not to affect the morning peak of HA:CO protein (Song *et al*, 2012), combining the four *prr* mutations greatly reduced CO protein and *FT* mRNA abundance in the morning (Fig 3I–L and Appendix Fig S2A, B, D and E). Finally, in the *prr9* mutant where the circadian clock is not strongly affected in light/dark cycles (Appendix Fig S2G; Ito *et al*, 2003; Farré *et al*, 2005), HA:CO protein abundance was strongly reduced in the morning when *PRR9* is expressed in WT plants (Figs 3I–L and EV3A). Another clock-controlled gene *TOE1* was reported to reduce CO protein abundance under LD (Zhang *et al*, 2015), and we found that its mRNA levels were increased in *toc1 prr5 prr7 prr9 SUC2::HA:CO*, suggesting that this might contribute to the observed reduction in CO protein abundance (Appendix Fig S2H and I). However, in *prr9 SUC2::HA:CO* where CO protein level is reduced specifically in the morning, *TOE1* mRNA level was not increased at this time (Appendix Fig S2H and I). Its mRNA level was, however, increased in *toc1 prr5 prr7 SUC2::HA:CO* in the morning where these *prr* mutations do not affect the abundance of CO protein (Appendix Fig S2H and I). These results indicate that changes in *TOE1* mRNA levels in the *prr* mutants are not linked to CO protein levels. Taken together, we conclude that the reduction of CO protein levels in *prr* mutants is not an indirect effect of altered expression of clock-output genes such as *FKF1* or *TOE1*, but rather reflects a more direct effect of PRRs on CO.

PRR proteins interact with CO

The above data support a direct effect of PRRs on HA:CO protein stabilization and therefore suggest that PRRs might physically interact with CO. To test this possibility fluorescence resonance energy transfer (FRET) and *in vivo* co-immunoprecipitation assays were performed. Plasmids carrying 35S::*CO:YFP* and 35S::*PRR:CFP* were transfected into *A. thaliana* protoplasts, and FRET between YFP and CFP was measured through acceptor photobleaching. All PRR:CFP proteins were localized in nuclei, and they were also co-localized with CO:YFP (Fig 5A). In the FRET assay, high FRET signals between CO:YFP and each PRR:CFP protein were detected (Figs 5A and B, and EV5B). As negative controls, plasmids containing transgenes that express CFP or YFP protein fused to AGAMOUS LIKE 16 (AGL16), a member of the MADS-box transcription factor family that was reported to localize to the nucleus, were transfected into protoplasts (Parenicova *et al*, 2003; Hu *et al*, 2014). Significantly lower FRET signals were observed between each PRR:CFP and AGL16:YFP or between AGL16:CFP and CO:YFP than between PRR:CFP and CO:YFP (Figs 5B and EV5B). The interaction between PRRs and CO was also tested in *Nicotiana benthamiana* leaves. *Agrobacterium* strains carrying 35S::*PRR:CFP* and 35S::*CO:YFP* constructs were infiltrated into *N. benthamiana* leaves and FRET between CFP and YFP measured. High FRET signals between CO:YFP and each PRR:CFP protein were detected (Fig EV5C and D). By contrast, significantly lower FRET signals were observed between each PRR:CFP and YFP alone, or between CFP alone and CO:YFP (Fig EV5D). Results obtained by the FRET analyses were then further tested by performing *in vivo* co-immunoprecipitation assays. Thus, PRR:GFP and HA:CO proteins were transiently expressed in *N. benthamiana* leaves and co-immunoprecipitation performed. *Agrobacterium* strains carrying 35S::*PRR:GFP* and 35S::*CO:HA* constructs were infiltrated into tobacco leaves. PRR:GFP was immunoprecipitated with an antibody against GFP and for each PRR a HA:CO co-immunoprecipitation could be observed (Fig 5C). By contrast, HA:CO was not precipitated by the GFP antibody when 35S:*HA:CO* was infiltrated alone or with 35S::*TRB3:YFP*, which expresses a fusion of TELOMERE REPEAT BINDING 3 fused to YFP (Figs 5C and EV5A; Schrumpfova *et al*, 2014; Zhou *et al*, 2016). In summary, these results indicate that each PRR can physically interact with CO *in planta*.

To determine the domain that mediates the physical interaction between CO and the PRRs, a series of *in vivo* immunoprecipitation assays were performed on truncated variants of CO fused to YFP, which were co-expressed with TOC1:HA in *N. benthamiana* (Fig 5D and E). TOC1:HA was most strongly co-immunoprecipitated with the YFP fusion protein containing the CCT domain of CO, indicating that TOC1:HA preferentially binds to this region of CO (Fig 5E). This domain was previously reported to be involved in mediating the binding of CO to DNA through its cognate binding site, defined as the CO response element (CORE) in the *FT* promoter (Tiwari *et al*, 2010). Moreover, the CCT domain was also defined as the region with which COP1 physically interacts (Jang *et al*, 2008; Liu *et al*, 2008).

Stabilization by the PRRs increases CO binding to *FT*

CO binds to a canonical CORE in the *FT* promoter, thereby enabling chromatin looping and enhanced *FT* transcription (Adrian *et al*, 2010; Tiwari *et al*, 2010; Song *et al*, 2012; Cao *et al*, 2014). Since

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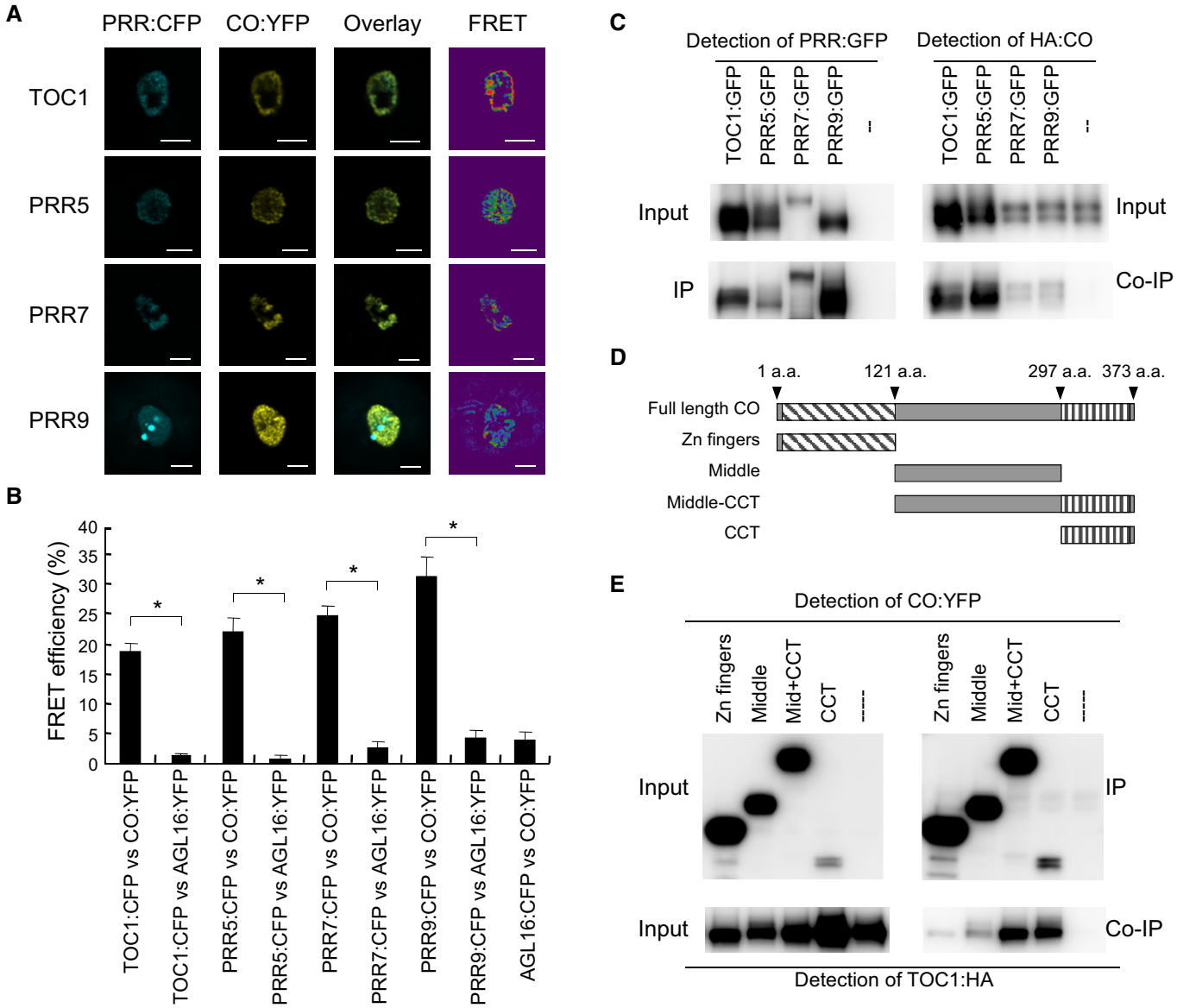

Figure 5. PRR proteins physically interact with CO protein *in planta*.

A, B Fluorescence resonance energy transfer (FRET) analysis between PRR:CFP and CO:YFP. PRR:CFP and CO:YFP were co-expressed in *A. thaliana* protoplasts, and FRET analyses were performed with 20 cells. Statistical significance was calculated using Student's *t*-test. *$P < 0.01$. Error bars indicate standard error within the 20 samples. Scale bars indicate 10 μm.

C *In vivo* immunoprecipitation assay with PRR:GFP and CO:HA protein. PRR:GFP and CO:HA were co-expressed in *N. benthamiana*, and co-immunoprecipitation assays performed.

D The schematic diagram of truncated CO proteins used for co-IP in (E).

E TOC1 preferentially binds to the CCT domain in CO. TOC1:HA was co-infiltrated with YFP fused to truncated versions of CO protein shown in (D) in *N. benthamiana*, and co-immunoprecipitation was performed.

Data information: For each experiment, two biological replicates were performed.

prr mutations strongly reduced the levels of HA:CO protein and *FT* transcripts in *SUC2*::*HA:CO* plants, we tested whether lower HA:CO protein levels in *toc1 prr5 prr7 prr9 SUC2*::*HA:CO* resulted in reduced binding of HA:CO to COREs at *FT*. To this end, a scan of the sequence of the entire intergenic up- and downstream region as well as the coding region of *FT* identified five canonical COREs. Chromatin immunoprecipitation (ChIP) was then performed in *SUC2*::*HA:CO* and *toc1 prr5 prr7 prr9 SUC2*::*HA:CO* plants to test binding of HA:CO at each of the 5 putative CO binding sites. In agreement with previous results, HA:CO was found to be strongly enriched at CORE1/2 in *SUC2*::*HA:CO* plants, whereas in *toc1 prr5 prr7 prr9 SUC2*::*HA:CO* quadruple mutant binding of HA:CO was strongly reduced, demonstrating a correlation between HA:CO protein abundance and its ability to bind to the CORE at *FT* (Fig 6A and B). Moreover, the ChIP experiment identified a previously unrecognized binding site for CO, CORE3, which is located in the

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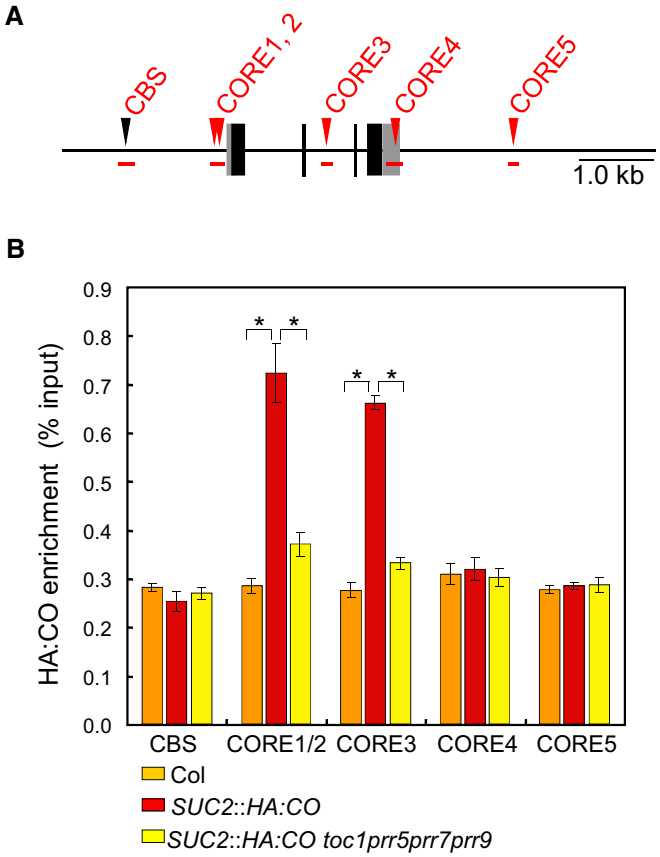

Figure 6. Occupancy of CO protein at the *FT* promoter is reduced in *prr* quadruple mutants.

A The location of each DNA fragment in the *FT* gene tested in ChIP assays. The amplicons are shown as red horizontal bars. The position of each predicted CO response element (CORE) is shown as red triangle.

B ChIP of CORE-containing amplicons within the *FT* gene with HA:CO protein. *SUC2::HA:CO*, *toc1 prr5 prr7 prr9 SUC2::HA:CO*, and Col were grown under LDs and harvested at ZT12. Enrichment of the CORE segments (CORE1-5 shown in A) was tested in each line. Error bars indicate standard error within three biological replicates. In each replicate, three technical replicates were performed. Statistical significance was obtained with Student's *t*-test, $*P < 0.01$.

second intron of *FT* and a reduction in enrichment of HA:CO was also detected at this CORE in *toc1 prr5 prr7 prr9 SUC2::HA:CO* plants (Fig 6A and B). In addition, ChIP experiments were performed using 35S::*TOC1 SUC2::HA:CO* plants, and HA:CO was found to be more strongly enriched at the COREs of *FT* in these plants compared to *SUC2::HA:CO* plants (Fig EV6). Although the effect of *TOC1* overexpression on HA:CO accumulation was not tested, these ChIP experiments are also consistent with our proposal that CO protein levels as determined by PRR activity are related to the amount of CO that is bound to COREs at *FT*.

Discussion

Perception of changes in day length enables plants to precisely determine the timing of the floral transition in their life cycle. In this process, a light-signaling pathway that transfers information on the light environment to a photoperiodic time-keeping mechanism is crucial. However, despite its significance, how information on light exposure is conveyed to the timing mechanism is not fully understood. In this study, we demonstrate that in *A. thaliana*, PRRs enable measurement of LDs by transferring information on light exposure to CO protein. This is achieved through the roles of PRRs in binding and stabilizing CO during the day to allow it to accumulate specifically under LDs. PRR paralogues contribute to detection of long-light periods through their diverged temporal expression patterns, resulting in CO accumulation in the morning and the evening specifically under LDs and thereby inducing *FT* transcription at these times. We propose that PRRs stabilize CO protein to promote *FT* transcription and flowering, conveying information on day length to the photoperiodic time-keeping mechanism and allowing accurate monitoring of LDs.

Novel roles for PRRs in controlling photoperiodic flowering

PRRs were originally identified as components of the circadian clock in *A. thaliana* and generally proposed to contribute to day-length measurement through control of the time-keeping mechanism associated with *CO* transcription (Strayer *et al*, 2000; Yanovsky & Kay, 2002; Ito *et al*, 2003, 2008; Yamamoto *et al*, 2003; Murakami *et al*, 2004; Nakamichi *et al*, 2007; Para *et al*, 2007). By contrast, we defined a previously unidentified role of these circadian-clock components in regulating CO activity, where they interact with and stabilize CO protein during the day, ensuring its accumulation under LDs. Therefore, specific circadian-clock components not only transfer temporal information to a photoperiodic time-keeping mechanism but also convey information on light exposure to the time-keeping mechanism, establishing measurement of day length.

In WT plants, *CO* mRNA accumulates in the morning and in the evening, coinciding with exposure to light and stabilizing CO protein at these times specifically under LDs. Such a temporal accumulation of CO protein is also observed in 35S::*CO* or *SUC2::HA:CO* lines where *CO* mRNA is expressed at similar levels throughout the day and is not regulated by the circadian clock (Fig 3I–L and Appendix Fig S3A and B; Valverde *et al*, 2004; Song *et al*, 2012). Here, several lines of experimental evidence indicate that PRRs are involved in this diurnal accumulation pattern of CO. The quantitative effect of the *prr9* mutation on HA:CO accumulation varied among experiments (Figs 3I–L and EV3A), but supported a role for PRR9 in conferring accumulation of CO specifically in the morning. Such a role was also consistent with the reduced *FT* mRNA levels observed in the morning in *prr9 SUC2::HA:CO* plants (Fig EV3B) and in the slightly late-flowering phenotype previously described for *prr9* mutants (Nakamichi *et al*, 2005). On the other hand, TOC1, PRR5, and PRR7 cause accumulation of CO in the evening, thereby illustrating how each PRR protein that acts in the central circadian-clock mechanism can also directly convey temporal information during the day to the photoperiodic timer CO. These PRR activities coincide with the morning and evening accumulation of *CO* mRNA in WT plants and enhance CO protein accumulation at these times specifically under LDs allowing CO to bind more strongly to *FT* (Fig 7). This elegant system directly couples CO stability and function in photoperiodic flowering to cycling circadian-clock components, ensuring that CO only binds to and activates *FT* transcription at

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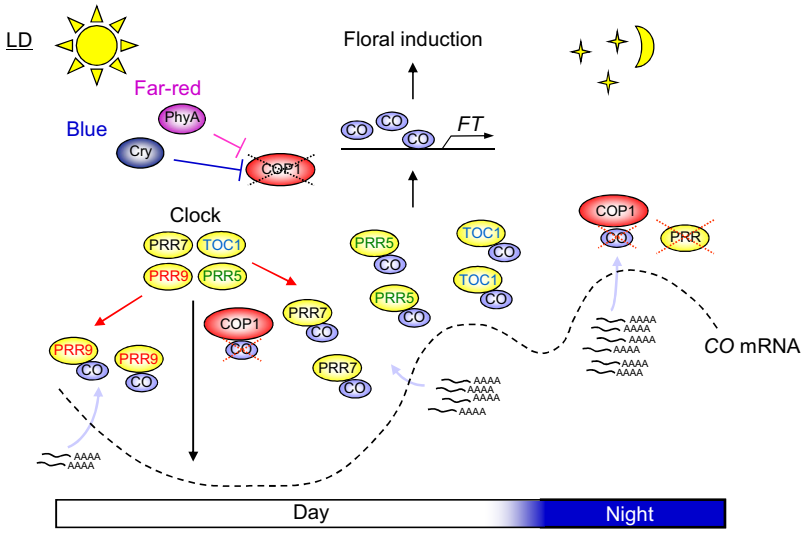

Figure 7. A model of day-length recognition through CO and PRRs.
PRRs control activity of the photoperiodic time-keeping mechanism through regulation of temporal abundance of *CO* mRNA under LDs and SDs. Under LDs, accumulation of *CO* transcripts coincides with exposure to light and PRRs function to stabilize CO protein during the day, thereby allowing it to accumulate under LDs and establishing recognition of LDs. In this mechanism, PRR9 mediates accumulation of CO protein in the morning, whereas TOC1, PRR5, and PRR7 mediate its accumulation in the evening. The mechanism of CO stabilization by PRRs is associated with their activities to suppress COP1 function to degrade CO. During the night under LDs, these stabilization mechanisms are lost because expression of PRRs is reduced, leading COP1 to target CO for degradation. CO protein stabilized by PRR during the day binds to the *FT* promoter to activate transcription of *FT* inducing flowering under LDs.

specific times during the day dependent on day length. Since CO and PRRs contain similar CCT DNA-binding domains (Matsushika *et al*, 2000; Gendron *et al*, 2012), PRRs might also bind along with CO to the *FT* promoter and contribute to the control of *FT* transcription, as was recently shown for the interaction between PRRs and PIF transcription factors on their target genes (Soy *et al*, 2016; Zhu *et al*, 2016). Morning *FT* expression is more pronounced when plants are exposed to shade (Wollenberg *et al*, 2008), suggesting that specific PRRs might also control this process by coupling a distinct flowering signal to CO protein stability or *FT* induction in the morning.

PRRs participate in light signaling that mediates photoperiodic flowering

The abundance of transcripts and proteins of PRRs exhibit circadian rhythms and under diurnal conditions they peak in expression sequentially at 2- to 3-h intervals during the light period in the order *PRR9*, *PRR7*, *PRR5,* and *TOC1* (Fujiwara *et al*, 2008). Overexpression of *PRR5* slightly affects the peak time of some PRRs under LDs, but mutations in *PRR* genes do not affect the peak time of expression of other *PRR*s under these conditions (Appendix Fig S4A and B). These proteins are degraded during the night, so they mainly accumulate during the day when CO protein also accumulates (Mas *et al*, 2003b; Farré & Kay, 2007; Ito *et al*, 2007; Kiba *et al*, 2007). Through this regulation, PRRs allow detection of long-light periods and contribute to recognition of LDs, interacting with and stabilizing CO protein during the day specifically under LDs.

COP1 is a key factor in controlling CO abundance by targeting it for degradation by the proteasome. Our genetic data using the quintuple mutant *cop1 prr9 prr7 prr5 toc1* in the *SUC2*::*HA:CO* background support the idea that *PRRs* suppress *COP1*-mediated degradation of CO during the day (Fig 4E and F). However, we also found that the levels of HY5 protein were not altered in *toc1 prr5 prr7 prr9* mutant (Fig 4H and I), suggesting that PRRs do not participate in mechanisms that control either *COP1* mRNA level, COP1 protein abundance or other mechanisms, including its light-dependent nucleocytoplasmic partitioning, that broadly regulate COP1 activity (Osterlund & Deng, 1998; Laubinger *et al*, 2006; Zuo *et al*, 2011). The long-hypocotyl phenotype of *toc1 prr5 prr7 prr9* mutant might suggest that PRRs prevent the function of *COP1* in the established light-signaling pathway (Fig EV4A). However, this long-hypocotyl phenotype may be due to increased accumulation of transcripts of the clock-controlled gene *PIF4*, which encodes a transcription factor involved in shade- and dark-mediated promotion of hypocotyl growth (Fig EV4B; Franklin, 2008; Nakamichi *et al*, 2009). Furthermore, PRRs physically bind to CO (Figs 5 and EV5), further decreasing the possibility that *PRRs* suppress general *COP1* activity or participate in the mechanisms that mediate light-dependent suppression of COP1 activity, such as promotion of its migration to the cytoplasm. Therefore, a more attractive hypothesis is that PRRs form a light-signaling mechanism dedicated to photoperiodic flowering through their accumulation during the day, transferring information on light exposure to CO protein. The pattern of accumulation of PRR proteins in the light is in part mediated by their degradation in the dark. TOC1 and PRR5 proteins are targeted for degradation by ZTL, which was originally identified as a component of the circadian clock (Somers *et al*, 2000; Mas *et al*, 2003b; Kiba *et al*, 2007). It is still not known how PRR7 and PRR9 proteins are degraded during the night.

Taken together, CO protein accumulation is likely to be controlled by a general light-signaling pathway involving COP1 and a dedicated signaling mechanism involving PRRs, which mainly function to degrade CO in the night and stabilize it in the day, respectively. However, COP1 clearly remains active at a reduced

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level during the day, triggering reduction of CO protein level at these times and lowering the abundance of CO under LDs (Jang *et al*, 2008). Our genetic data using the quintuple mutant *cop1 prr9 prr7 prr5 toc1* in the *SUC2*::*HA:CO* background clearly support the idea that *PRRs* suppress *COP1*-mediated degradation of CO during the day (Fig 4E and F). Therefore, although PRRs may not control general *COP1* activity or participate in light-dependent migration of COP1 to the cytoplasm, they may function in a more specific way to reduce COP1 activity on CO during the day and thereby limit this activity to darkness. In turn, this would ensure sufficient CO protein accumulation under LDs at the specific times that the PRRs are expressed and confer appropriate timing of floral induction under these conditions. The precise molecular mechanism by which PRRs suppress CO degradation by COP1 is still unclear. An attractive hypothesis is that since both PRR and COP1 proteins bind to CO protein, suppression of CO degradation by PRRs might occur at the level of protein–protein interaction. For example, PRRs might interact with CO to directly interfere with COP1 binding and thereby reduce CO degradation. Our co-immunoprecipitation data showing that TOC1 preferentially binds to the CCT domain in CO (Fig 5D and E) is consistent with this hypothesis, since this domain is also responsible for interaction with COP1 (Jang *et al*, 2008; Liu *et al*, 2008). Alternatively, PRRs might act specifically in the phloem companion cells of the vasculature where CO is expressed to directly affect COP1 activity by mechanisms similar to those observed in the general light-signaling pathways. According to this model, in our study the effect of *toc1 prr5 prr7 prr9* mutations on HY5 levels in the vasculature would not be detected because HY5 is broadly expressed (Oyama *et al*, 1997). However, in this case, since PRRs are also broadly expressed in *A. thaliana* to control circadian-clock functions (Para *et al*, 2007; Fujiwara *et al*, 2008), the activity of PRRs to directly control COP1 activity might be expected to occur in other tissues as well.

In addition to *COP1* and *FKF1* that were discussed above, *HOS1* and *TOE1* have also been implicated in regulating CO stability (Lazaro *et al*, 2012; Zhang *et al*, 2015). Although it is possible that HOS1 and TOE1 might also be regulated by PRRs, our genetic data with the *cop1 toc1 prr5 prr7 prr9* quintuple mutant do not currently support this hypothesis, because the *cop1* mutation is epistatic to the *prr* mutations with respect to CO protein abundance (Fig 4E and F). If the PRRs do suppress other mechanisms that regulate CO protein abundance independently of COP1, such as those proposed to act through *HOS1* or *TOE1* (Lazaro *et al*, 2012, 2015; Zhang *et al*, 2015), then their increased activity by the *prr* mutations would be expected to reduce the level of CO protein even in the *cop1* mutant background.

Alleles of *PRR* genes contribute to QTLs for flowering time in a wide range of crop species. *Ppd-H1*, *Ppd-1*, *BOLTING TIME CONTROL 1 (BvBTC1)*, *PRR37,* and *SbPRR37* encode PRR proteins in barley, wheat, beet, rice, and sorghum, respectively, and allelic variation at these genes confers natural diversity in flowering time (Turner *et al*, 2005; Pin *et al*, 2009; Murphy *et al*, 2011; Campoli *et al*, 2012; Shaw *et al*, 2012; Koo *et al*, 2013). Interestingly, in contrast to *A. thaliana* PRRs that affect the time-keeping mechanism associated with *CO* transcription, the *PRRs* identified in several crop species have weak or no effects on this timing mechanism, despite their strong effects on *FT* transcription (Turner *et al*, 2005; Campoli *et al*, 2012; Koo *et al*, 2013). In this study, we demonstrated a novel function of PRRs in *A. thaliana*, where they bind to and stabilize CO during the day to enable it to accumulate specifically under LDs and initiate floral transition. Whether these roles for PRRs are also conserved in other plants including crop species is an important issue to resolve. Further molecular-genetic analyses using these crops may help us to understand how changes in the mechanisms of day-length recognition facilitate adaptation to different latitudes in crops and has potential to improve performance in different environments.

Materials and Methods

Plant materials and growth conditions

We used *toc1-101*, *prr5-11*, *prr7-11*, and *prr9-10* as *prr* mutants (Ito *et al*, 2003; Yamamoto *et al*, 2003; Salome *et al*, 2008). *SUC2*::*CO* and *SUC2*::*HA:CO* have been previously described (An *et al*, 2004; Jang *et al*, 2009). All of the single, double, triple, and quadruple *prr* mutants with *SUC2*::*CO* and *SUC2*::*HA:CO* were generated by genetic crossing. 35S::*TOC1* was crossed to *SUC2*::*HA:CO* and used for ChIP experiments. For *cop1* mutant analyses, *cop1-4* was used (McNellis *et al*, 1994). For *fkf1* mutant analyses, *fkf1-2* was used (Imaizumi *et al*, 2003). *SUC2*::*HA:CO fkf1-2* was generated by genetic crossing. *TMG*, *pPRR*5::*PRR5:GFP*, *pPRR7*::*FLAG:PRR7:GFP*, and *pPRR9*::*FLAG:PRR9:GFP* plants have been previously described (Mas *et al*, 2003a; Fujiwara *et al*, 2008; Nakamichi *et al*, 2010). For plasmid construction of *pCO*::*HA:CO*, 35S promoter in the pAlligator2 vector was replaced by 2.5 kb *CO* promoter. Primers for amplifying the *CO* promoter are GCGGCTCTAGATTTTGATCATGCAACATAAACTTA TAAGC and CGGCCCTCGAGAATAACTCAGATGTAGTAAGTTTGAT GGTG. Full-length *CO* cDNA was inserted into the vector by recombination following Invitrogen instructions. *Arabidopsis thaliana* transformation was performed by floral dip using *Agrobacterium*. GFP-positive seeds were selected using a Leica MZFLIII stereomicroscope equipped with GFP filters.

For cDNA and protein analyses, plants were grown for 9 days on MS agar plate. In all expression analyses (except Fig 4A–D), FRET, co-IP, and ChIP experiments plants were grown in climatic chambers with fluorescent lamps at 22°C. In experiments in Fig 4A–D, plants were grown in climate chambers with LED light of blue or far-red at 22°C. For flowering-time analyses, plants were grown on soil at 21°C under 100–150 μmol m^{-2} s^{-1} photosynthetic active radiation. The number of total leaves and the number of days after sowing when flower bolts were 2 cm in length was used as a measure of flowering.

RNA isolation, cDNA analysis, and expression data analyses

RNA was isolated from 50 to 100 *A. thaliana* seedlings for each line grown on an agar plate by RNeasy Plant Mini Kit (Qiagen) with on-column DNAase treatment. cDNA was synthesized by Superscript II reverse transcriptase (Life Technologies). Expression analyses were performed by real-time PCR (Roche LightCycler). Primers used for the expression analyses are *CO* (GCATGTGTCACAACAGCTTCAC and ATGCCTTCCTCGAAGCATACC), *FT* (TCAGAGGGAGAGTGGC TG and TCACCGTTCGTTACTCGTATC), *PP2a* (CTTGGTGGAGCTAA GTGAAGACC and CGCCCAACGAACAAATCACAGA), *PIF4* (TCCGA CCGGTTTGCTAGA and ACCTAGTGGTCCAAACGAGAA), *TOE1* (GT AACTGGGGATGGCAGAGA and TGTCCCCTTACAAGTTAAGGGT), *TOE2* (TCTCATGAACCGCCCACAA and TGTGCTGAGCCAAACGAT

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GA), and *PRR9* (CCTCGAGTGAAAGGCCAGT and CAAAAGTTGCC CCAGTATCTCA).

For expression data analyses including both mRNA and proteins, the values of the expression levels in two or three biological data sets were averaged and combined. Prior to this process, the values of expression levels in each data set were pre-normalized to the highest value in each data set, which was given the value of 1.

Analysis of HA:CO protein abundance

50–100 *Arabidopsis* seedlings for each line grown on an agar plate were ground in liquid nitrogen. Approximately 500 μl of leaf powder was suspended with the nuclear isolation buffer (20 mM Tris–HCl, 20 mM $MgCl_2$, pH 6.8, 5% sucrose, 40% glycerol, 0.8% Triton X-100, 0.08% β-mercaptoethanol, 0.2% SIGMA plant protease inhibitor, 1 mM DTT, 1.3 mM PMSF). The samples were centrifuged at 3,000 *g* for 5 min, and the supernatant was removed. The pellets were washed four times with the nuclear isolation buffer with the same procedure. The pellets were suspended in the SDS sample buffer and thereafter heated at 95°C for 10 min, and 20 μl of the supernatants were loaded on acrylamide gel. For Western analyses, anti-HA peroxidase high affinity (Roche) was used for detecting the HA:CO signal. Anti-histone 3a was used for detecting the H3a signal as loading control. Signals were detected by LAS-4000 (Fujifilm) and measured by the ImageJ software.

MG132 treatment and induction of HA:CO accumulation

Approximately 30 seedlings for each line were grown on MS agar media for 10 days and transferred at ZT5 to MS liquid media containing either 100 μM of MG132 or only the DMSO solvent. Samples were vacuum infiltrated for 10 min, transferred back to the growth cabinet with the liquid media, and harvested at ZT8. Nuclear protein was extracted with the method described above. HA:CO and H3a signals were detected by Western blot with antibodies indicated in the main method section.

FRET and co-IP experiments

For FRET in *A. thaliana* cells, protoplasts from adult leaves of plants grown under 12-h light/12-h dark were isolated by previously described methods (Yoo *et al*, 2007; Wu *et al*, 2009). In these experiments, 9 μg of plasmids carrying 35S::*PRR:CFP* or 35S::*CO:YFP* was used for transfection to ~100 000 protoplasts. After transfection, the protoplasts were placed in a growth chamber with 12-h light/12-h dark with the low intensity of light and the next day FRET was performed. 20 and 10 cells for the first and second experiments, respectively, were imaged with the confocal laser scanning microscope SP8 (Leica), and after bleaching of the acceptor (CO:YFP), the change in donor (PRR:CFP) fluorescence was quantified by comparing pre- and post-bleaching images. FRET efficiency was calculated with a formula "(pre-bleaching – post-bleaching)/pre-bleaching". For FRET using *N. benthamiana* leaves, *Agrobacterium* containing 35S::*PRR:CFP* or 35S::*CO:YFP* was infiltrated into *Nicotiana benthamiana*, and after 3 days, protein–protein interaction was measured by CFP-YFP FRET by acceptor photobleaching. 30 and 10 *Nicotiana benthamiana* cells for the first and second experiments, respectively, were imaged with LSM 780 (Carl Zeiss), and after bleaching of the acceptor (CO:YFP), the change in donor (PRR:CFP) fluorescence was quantified by comparing pre- and post-bleaching images.

For coIP experiment, *Agrobacterium* containing 35S::*PRR:GFP* or 35S::*HA:CO* was infiltrated into *Nicotiana benthamiana*, and after 3 days, the infiltrated leaves were harvested. The leaves were ground in liquid N_2, and ~500 μl of the leaf powder was suspended in the nuclear isolation buffer (50 mM Tris–HCl pH 7.4, 0.5% NP-40, 40% glycerol, 1 mM DTT, 0.2% SIGMA plant protease inhibitor, 1.3 mM PMSF, 50 μM MG132). The samples were centrifuged at 1,000 *g* for 5 min, and the supernatants were removed. Pellets were washed four times with the nuclear isolation buffer using the same procedure. After removing the supernatant, the pellets were suspended in 0.3 ml of the sonication buffer (50 mM Tris–HCl pH 7.4, 50 mM NaCl, 0.5% NP-40, 1 mM DTT, 0.2% SIGMA plant protease inhibitor, 1.3 mM PMSF, 50 μM MG132), and the samples were sonicated for 2.5 min, 5 min, or 7.5 min for PRR9, PRR5/PRR7, or TOC1, respectively, with a bath sonicator (DIAGENODE BIORUPTOR) with "high" setting for 15 min with 15 s sonications separated by 15 s breaks to release PRR:GFP and HA:CO proteins from the nuclei. 0.7 ml of the sonication buffer and 20 μl of 5 M NaCl were added to the samples, and the samples were incubated for 15 min with mild agitation. The samples were centrifuged at 21,000 *g* for 15 min, and the supernatants were recovered. Immunoreaction was performed by adding the anti-GFP antibody (Roche) to the supernatants and incubating these for 1 h, and afterward protein-G sepharose (Roche) was added and the samples were incubated for 3 h. The sepharose was washed four times with the washing buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.5% NP-40, 1 mM DTT). The sepharose beads was incubated with 40 μl of the SDS sample buffer and heated at 95°C for 10 min, and a half of the supernatant was loaded on SDS acrylamide gel. For input samples, 7.5% of the sonicated protein samples were loaded on the gel. GFP and HA signals were detected with the anti-GFP antibody (Abcam) and the anti-HA peroxidase (Roche), respectively. For the co-IP experiments between TOC1:HA and truncated CO:YFPs, 1–121 a.a. for the zinc fingers, 122–297 a.a. for the middle part, 122–373 a.a. for the middle-CCT, and 298–373 a.a. for the CCT were fused to YFP.

ChIP assay

ChIP was performed as published previously (Hyun *et al*, 2016) with minor modifications. Plants were grown for 10 days in LDs and harvested at *Zeitgeber* time (ZT) 12. Before plant tissue was cross-linked in 1% formaldehyde for 10 min, plants were infiltrated with 1 μM DSG for 10 min in a vacuum desiccator. Chromatin immunoprecipitation of HA:CO for *toc1 prr5 prr7 prr9 SUC2*::*HA:CO* and *35S*::*TOC1 SUC2*::*HA:CO* was performed by using HA antibody (Abcam, ab9110). ChIP-qPCR was analyzed, and the relative enrichment of the IP/Input at each genomic region tested was normalized to that of the reference locus, *ACT8*.

Expanded View for this article is available online.

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Author contributions

LS-K performed a part of the experiments on flowering-time measurement and co-IP, as well as the experiment for checking *SUC2* gene expression. LS-K also prepared the *A. thaliana* p*CO::HA:CO* line that SJ previously created through vector construction and transformation. RR performed all the ChIP experiments. VF performed the experiment for checking effect of MG132 treatment on HA:CO accumulation. RH carried out all other experiments. GC and RH wrote this article.

Conflict of interest

The authors declare that they have no conflict of interest.

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