

# *Arabidopsis* COP1 shapes the temporal pattern of CO accumulation conferring a photoperiodic flowering response

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The transcriptional regulator CONSTANS (CO) promotes flowering of *Arabidopsis* under long summer days (LDs) but not under short winter days (SDs). Post-translational regulation of CO is crucial for this response by stabilizing the protein at the end of a LD, whereas promoting its degradation throughout the night under LD and SD. We show that mutations in CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1), a component of a ubiquitin ligase, cause extreme early flowering under SDs, and that this is largely dependent on CO activity. Furthermore, transcription of the CO target gene *FT* is increased in *cop1* mutants and decreased in plants overexpressing *COP1* in phloem companion cells. COP1 and CO interact *in vivo* and *in vitro* through the C-terminal region of CO. COP1 promotes CO degradation mainly in the dark, so that in *cop1* mutants CO protein but not CO mRNA abundance is dramatically increased during the night. However, in the morning CO degradation occurs independently of COP1 by a phytochrome B-dependent mechanism. Thus, COP1 contributes to day length perception by reducing the abundance of CO during the night and thereby delaying flowering under SDs.

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## Introduction

Exposure to light influences many aspects of the plant life cycle, a process referred to as photomorphogenesis. Light promotes seed germination and seedling growth, thereby ensuring that young plants are exposed to an optimal environment for photosynthesis. Photomorphogenesis also has important functions in the development of adult plants (Neff *et al*, 2000). The mechanisms controlling adult photomorphogenic traits such as control of flowering in response to day length are less well understood than those that occur in the seedling. However, a genetic pathway that promotes flowering of *Arabidopsis* in response to long days (LDs) has been defined (Searle and Coupland, 2004; Imaizumi and Kay, 2006). Within this pathway, the transcriptional regulator CONSTANS (CO) has an important function by promoting flowering specifically under LDs. Here, we demonstrate that the ubiquitin ligase CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1), a major regulator of seedling photomorphogenesis (Deng *et al*, 1992), negatively regulates CO protein abundance in the vascular tissue of adult plants as part of the mechanism by which *Arabidopsis* discriminates between LD and SD during flowering-time control.

CO is a major regulator of photoperiodic flowering. Mutations in *CO* delay flowering specifically under LD, whereas its overexpression from a viral promoter causes extreme early flowering under LD and SD. CO contains two B-box-type zinc-finger motifs near its N terminus and a CCT (CONSTANS, CONSTANS-LIKE, TOC1) domain at its C terminus (Putterill *et al*, 1995). The latter domain is plant specific, but shows similarity to the DNA-binding domain of the HAP2 subunit of the CCAAT box-binding complex, suggesting that CO might bind to DNA directly (Wenkel *et al*, 2006). The closely related genes *FLOWERING LOCUS T (FT)* and *TWIN SISTER OF FT (TSF)* are highly and rapidly increased in expression in response to CO expression (Samach *et al*, 2000; Wigge *et al*, 2005; Yamaguchi *et al*, 2005). These genes encode RAF kinase inhibitor-like proteins that exert an effect as potent inducers of flowering (Kardailsky *et al*, 1999; Kobayashi *et al*, 1999). CO activates *FT* in the companion cells of the phloem within the vascular tissue, and FT protein is then proposed to move through the phloem sieve elements to the shoot apical meristem (An *et al*, 2004; Corbesier *et al*, 2007; Jaeger and Wigge, 2007; Mathieu *et al*, 2007), where it changes gene expression patterns and induces flowering (Abe *et al*, 2005; Wigge *et al*, 2005; Searle *et al*, 2006).

The mechanism by which CO activity is controlled by day length involves both transcriptional and post-translational regulation. CO transcription is regulated by the circadian

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clock so that its expression rises around 12 h after dawn and stays high until the following dawn (Suarez-Lopez *et al*, 2001). Exposure to light between 10 and 14 h after dawn further promotes CO transcription through the activity of the photoreceptor FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1) and its interacting partner GIGANTEA (GI) (Suarez-Lopez *et al*, 2001; Imaizumi *et al*, 2003; Sawa *et al*, 2007). At the post-translational level, CO protein is stabilized when plants are exposed to light, whereas in darkness CO protein is rapidly degraded through ubiquitination and the activity of the proteasome. These mechanisms combine to ensure that a peak in CO protein abundance occurs under LDs when plants are exposed to light between 10 and 16 h after dawn, whereas under SD, when plants are exposed to darkness during this interval, CO protein does not accumulate (Valverde *et al*, 2004). The importance of ubiquitination and degradation of CO protein by the proteasome in these processes was demonstrated by use of proteasome inhibitors. These regulatory steps ensure that transcription of *FT* and *TSF* occurs under LDs but not under SDs.

The photoreceptors required for post-translational regulation of CO have been characterized. Mutations in the genes encoding the photoreceptors phytochrome A (*phyA*) and cryptochrome 2 (*cry2*) delay flowering, and these mutations also reduce the accumulation of CO protein (Valverde *et al*, 2004). Similarly, far-red light or blue light promotes flowering and stabilizes CO protein, and these regions of the spectrum activate *phyA* and *cry2*, respectively. In contrast, red light delays flowering and reduces the accumulation of CO protein. This response appears to be mainly controlled by phytochrome B (*phyB*), because *phyB* mutations cause early flowering and allow increased accumulation of CO protein.

COP1 is a major negative regulator of photomorphogenic responses, so that *cop1* mutants undergo photomorphogenesis in darkness in the absence of photoreceptor activation (Deng *et al*, 1991). *COP1* encodes a RING finger protein with a coiled-coil motif and WD40 repeats (Deng *et al*, 1992), and exerts an effect as a ubiquitin ligase that promotes the degradation of transcription factors implicated in seedling photomorphogenesis (Osterlund *et al*, 2000). In mammalian and plant cells, COP1 seems to exert an effect as part of a complex that also includes DEETIOLATED 1 (DET1), DAMAGED DNA-BINDING PROTEIN 1 (DDB1), cullin 4A and RING BOX 1 (RBX1) (Chory *et al*, 1989; Schroeder *et al*, 2002; Wertz *et al*, 2004; Hoecker, 2005; Chen *et al*, 2006). However, the SUPPRESSOR OF PHYTOCHROME A-105 1 (SPA) family of proteins is plant specific, related in sequence to COP1 and modulates the ubiquitin ligase activity of COP1. SPA proteins contain a coiled-coil domain and WD40 repeats related to those of COP1 as well as a kinase-like domain not present in COP1 (Hoecker *et al*, 1999). Quadruple mutants in which the four SPA genes are mutated exhibit a phenotype similar to that of *cop1* mutants (Laubinger *et al*, 2004). Furthermore, SPA1 and COP1 physically interact and SPA1 modulates COP1 activity *in vitro* (Hoecker and Quail, 2001; Saijo *et al*, 2003; Seo *et al*, 2003).

Several protein targets for COP1 are transcription factors that regulate seedling photomorphogenesis (Osterlund *et al*, 2000; Seo *et al*, 2003; Duek *et al*, 2004; Jang *et al*, 2005; Yang *et al*, 2005). Each of these proteins was shown based on mutagenesis studies to have a function in the regulation of seedling growth in response to light. COP1 targets each of these

proteins for degradation in the dark, but in the light COP1 activity is suppressed allowing these transcription factors to accumulate and promote seedling photomorphogenesis.

In addition to these roles in seedling development, COP1 also influences photomorphogenesis of adult plants. Although null mutant alleles of *COP1* cause seedling lethality, plants homozygous for weaker *cop1* alleles are viable. These plants are early flowering, particularly under SDs, indicating that COP1 is required for the suppression of flowering (McNellis *et al*, 1994). Furthermore *cop1* mutants, but not wild-type (WT) plants, flower in darkness if provided with sugar (Nakagawa and Komeda, 2004). In addition, *spa1* mutants flower early and SPA proteins modulate CO abundance so that in *spa1 spa3 spa4* triple mutants 16 h after dawn under LDs increased levels of CO protein were detected (Ishikawa *et al*, 2006; Laubinger *et al*, 2006). Here, we analysed the role of COP1 in the light regulation of flowering time by genetic and molecular studies. We show that COP1 represses CO activity in the vascular tissue, and reduces CO protein levels particularly under SDs and in the dark, thereby facilitating a flowering response to day length.

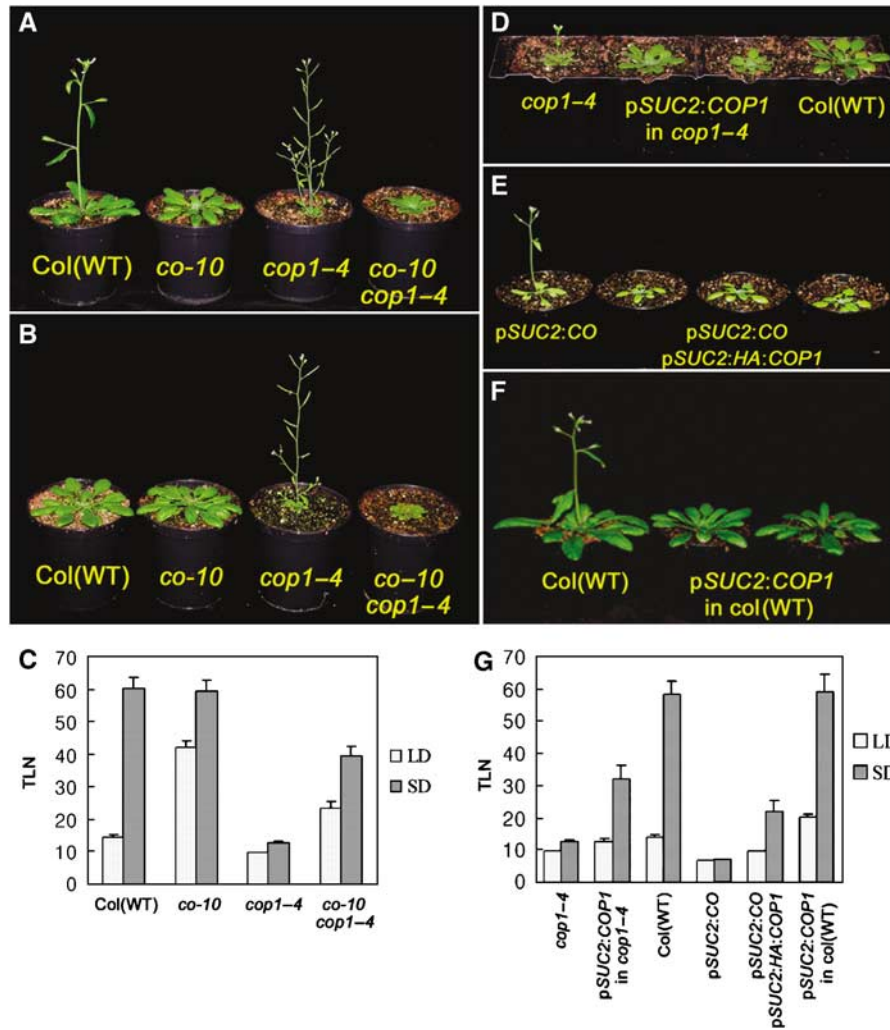
## Results

### **Genetic and spatial interactions between COP1 and CO in the regulation of flowering time**

Previously *cop1* mutants were shown to flower earlier than WT plants under short days (SDs) and at a similar time to WT plants under LDs (McNellis *et al*, 1994). Under our conditions, *cop1-4* mutants flowered dramatically earlier than WT plants under SDs, as shown previously, but in addition flowered earlier than WT plants under LDs. The *cop1-4* mutant produced around 53 leaves fewer than WT plants before flowering under SDs, whereas under LDs the difference between mutant and WT was around 5 leaves (Figure 1A–C; Supplementary Table 1). Therefore, the photoperiod response of *cop1-4* mutants was severely reduced so that they flowered after forming only 3 leaves more under SDs than LDs, whereas WT plants formed around 45 leaves more under SDs.

In WT plants, CO promotes early flowering under LDs but not SDs. To test whether the early flowering of *cop1-4* mutants under SDs was caused by activation of CO under these conditions, the *cop1-4 co-10* double mutant was constructed and its flowering time was measured. The double mutant flowered after forming around 30 leaves more than *cop1-4* mutants under SDs, demonstrating that CO has an important role in the early flowering of *cop1-4* mutants under SDs (Figure 1B and C). Nevertheless, *cop1-4 co-10* plants formed 20 leaves fewer than *co-10* plants under these conditions, indicating that part of the early flowering of the *cop1-4* mutant occurs independently of CO. Under LDs, *cop1-4 co-10* plants also flowered at a time intermediate between *co-10* and *cop1-4* (Figure 1A and C). These genetic results suggest that COP1 exerts an effect as a negative regulator of CO under SDs, so that CO promotes flowering of *cop1-4* mutants but not WT plants under SDs.

CO is expressed only in the vascular tissue and exerts an effect in the phloem companion cells to activate the transcription of the flowering-time gene *FT* (Takada and Goto, 2003; An *et al*, 2004). To test whether COP1 also regulates flowering from the phloem, COP1 or HA:COP1 was expressed from the



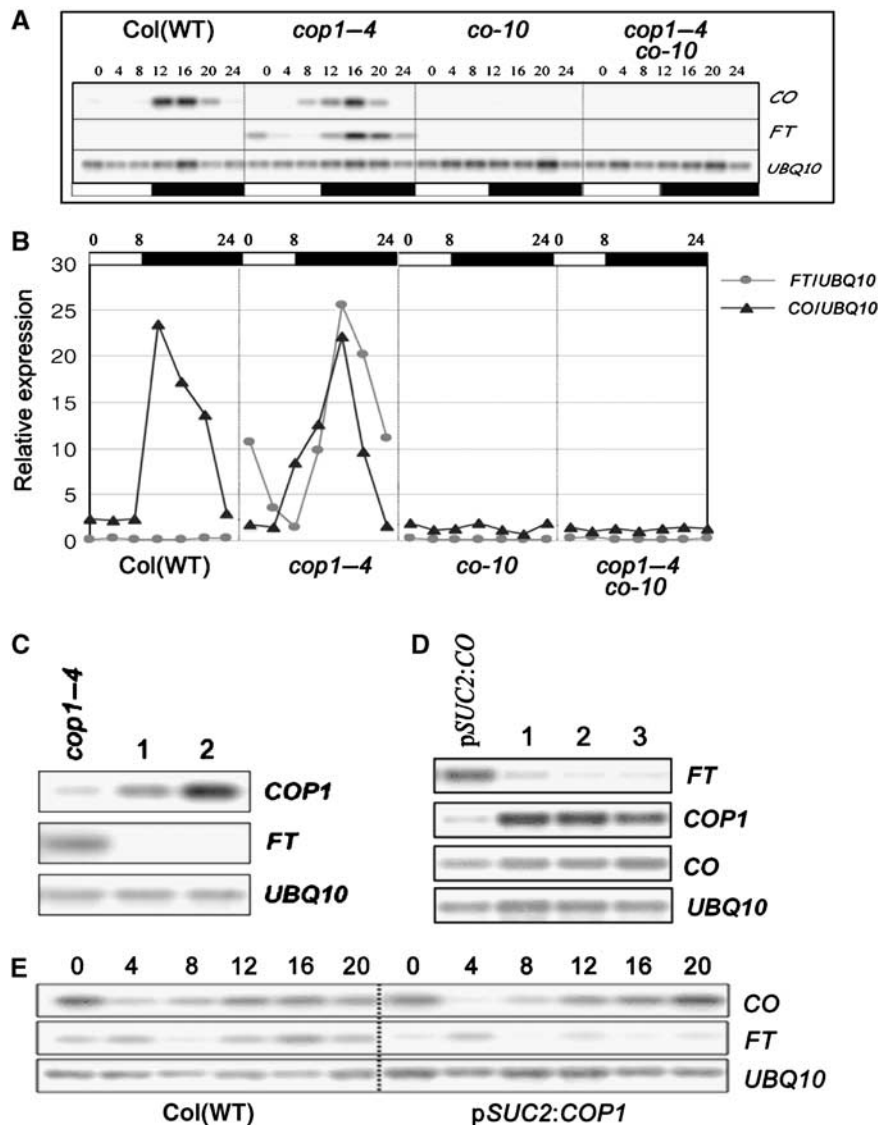
**Figure 1** Genetic characterization of the interaction between CO and COP1. (A, B) *cop1-4* mutants flowered earlier than wild-type Columbia plants irrespective of photoperiod, and the *co-10* mutation suppresses the extreme effect of the *cop1-4* mutation on flowering time under 16 h LD (A) and 8 h SD (B). (C) Flowering times in LD and SD of genotypes shown in (A, B). Flowering time is expressed as total leaf number (TLN) at flowering. (D) *COP1* expression under the phloem-specific promoter *SUC2* largely rescued the early-flowering *cop1-4* mutant phenotype. The plants were grown under SD. (E) Simultaneous expression of CO and COP1 in the phloem tissue. *SUC2:CO SUC2:HA:COP1* transgenic plants flowered later than *SUC2:CO* transgenic plants. (F) *SUC2:COP1* caused late flowering of wild-type Columbia plants under LD. (G) Flowering times expressed as TLN at flowering under LD and SD of genotypes shown in (D-F).

*SUC2* promoter, which is active specifically in the phloem companion cells (Imlau *et al*, 1999). The *SUC2:COP1* transgene was introduced into WT Columbia plants and into *cop1-4* mutants, whereas *SUC2:HA:COP1* was introduced into *SUC2:CO* plants. *SUC2:COP1* delayed flowering of *cop1-4* mutants under LDs and SDs, and of WT plants under LDs (Figure 1D-G). Therefore, COP1 exerts an effect in the companion cells, where CO is expressed, to delay flowering. However, *SUC2:COP1 cop1-4* plants still flower earlier than WT plants under SDs, suggesting that COP1 expression in companion cells is not sufficient to completely rescue the early-flowering phenotype of *cop1-4* mutants, and therefore that COP1 probably also exerts an effect in additional cell types to delay flowering. The observation that *SUC2:HA:COP1* delays flowering of *SUC2:CO* plants under LDs and SDs supports the idea that the delay of flowering associated with *SUC2:COP1* is at least in part caused by reduction of CO activity (Figure 1G). Taken together, the flowering-time phenotypes of plants misexpressing COP1 in the phloem are consistent with the idea that COP1 exerts an

effect in the phloem companion cells to repress the promotion of flowering by CO.

### COP1 reduces FT mRNA levels

*FT* transcription is activated by CO and likely represents a direct target of CO protein (Samach *et al*, 2000; Wigge *et al*, 2005). Therefore, if COP1 exerts an effect to repress CO activity this should be reflected in reduced *FT* mRNA levels. In WT plants grown under SDs, CO mRNA is present during the night but *FT* mRNA is not expressed, because CO protein is rapidly degraded in the dark (Suarez-Lopez *et al*, 2001; Valverde *et al*, 2004). The effects of COP1 on CO transcription and CO activity were tested by analysing CO and FT mRNA levels at 4-h intervals for 24 h in SD-grown plants of different genotypes (Figure 2A and B). In WT Columbia, CO mRNA was detected during the night under SDs, but was absent in *co-10* and *cop1-4* *co-10* plants as expected due to the T-DNA insertion present in the CO gene in the *co-10* allele (Materials and methods). In *cop1-4* mutants, the CO mRNA pattern is similar to that observed in WT plants but rises earlier,



**Figure 2** Effect of COP1 on *CO* and *FT* mRNA levels. (A) *CO* and *FT* mRNA analysis in wild-type (WT) Columbia, *cop1-4* mutants, *co-10* mutants and *cop1-4 co-10* double-mutant plants under 8 h SDs. (B) Quantification of the mRNA levels shown in (A). Expressed as a ratio between *UBQ10* mRNA level and *FT* or *CO* mRNA level. (C) *COP1* and *FT* mRNAs in *cop1-4* mutants and two *SUC2:COP1 cop1-4* transformants. All plants were grown under SD and harvested 16 h after dawn. (D) *FT*, *COP1* and *CO* mRNAs in *SUC2:CO* and three *SUC2:CO SUC2:HA:COP1* transformants. All plants were grown under SD and harvested 8 h after dawn. (E) *CO* and *FT* mRNAs in WT Columbia plants and in a *SUC2:COP1* Columbia transformant. All plants were grown under LD and harvested at 4-h intervals. All genotypes are in the accession Columbia, and in (C, D) the numbers represent independent transgenic plants. In (A, E) 2-week-old seedlings were sampled, whereas in (C, D) rosette leaves of 3-week-old plants were harvested.

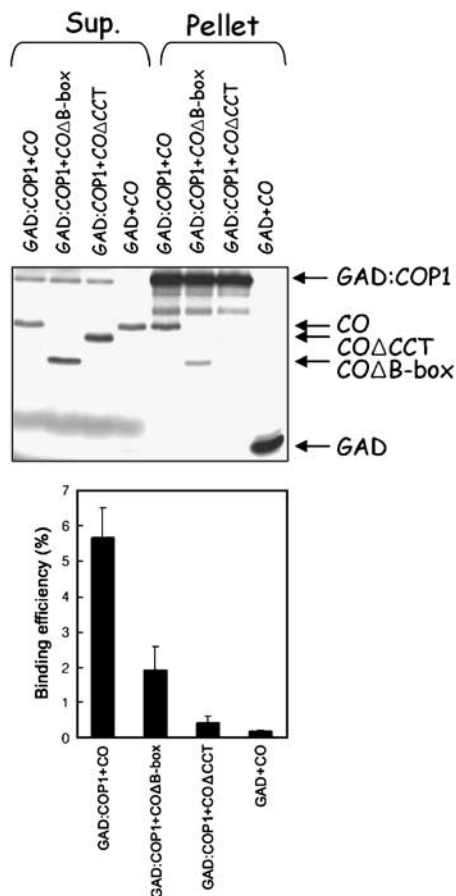
appearing weakly 8 h after dawn, whereas in WT plants *CO* mRNA was first detected 12 h after dawn. In contrast, *FT* mRNA was detected in *cop1-4* mutants but not WT plants, consistent with the early flowering of these mutants under SDs. Similarly, under LDs, *FT* mRNA levels were much higher in *cop1-4* plants than in WT plants consistent with the earlier flowering of the mutants under these conditions (Supplementary Figure 1). However, under LDs, *CO* mRNA was consistently detected at lower levels in *cop1-4* mutants than in WT plants (Supplementary Figure 1). The expression of *FT* mRNA in *cop1-4* mutants requires *CO* activity, as demonstrated by the absence of *FT* mRNA in *cop1-4 co-10* plants (Figure 2A). These results are consistent with the idea that COP1 delays flowering of WT plants under SDs, and to a lesser extent under LDs, by repressing *CO* activity and thereby preventing *FT* expression.

The abundance of *FT* mRNA was also tested in transgenic plants expressing *COP1* or *HA:COP1* mRNAs at high levels in the phloem companion cells from the *SUC2* promoter (Figure 2C–E). *CO* and *FT* mRNA levels were compared through a LD time course in *SUC2:COP1* and WT plants. *CO* mRNA levels were very similar in both genotypes at all times, whereas *FT* mRNA levels were severely reduced in *SUC2:COP1* plants (Figure 2E), consistent with the overexpression of COP1 in phloem companion cells leading to a reduction in *CO* activity at the post-transcriptional level. Similarly, 16 h after dawn under SDs, when *FT* mRNA reaches peak levels in *cop1-4* mutants (Figure 2A), *SUC2:COP1 cop1-4* plants displayed severely reduced levels of *FT* mRNA (Figure 2C). Finally, in *SUC2:HA:COP1 SUC2:CO* plants the level of *FT* mRNA was lower than in the *SUC2:CO* progenitor plants, but the level of *CO* mRNA was unaffected (Figure 2D).

Therefore, analysis of *FT* mRNA in these transgenic plants supports the conclusion that COP1 delays flowering by repressing at the post-transcriptional level the capacity of CO to promote *FT* transcription in the phloem companion cells.

### COP1 and CO physically interact *in vitro* and *in vivo*

The observation that the ubiquitin ligase COP1 represses CO-mediated activation of *FT* suggested that CO might be a substrate for COP1. To test this hypothesis, we first investigated whether COP1 was able to physically interact with CO. In the yeast two-hybrid system, we detected no interaction between CO and COP1, although an interaction between COP1 and the CO-related protein CO-LIKE3 (COL3) was previously detected by this method (Datta *et al*, 2006), and we were able to confirm this interaction. Therefore, whether COP1 and CO interact *in vitro* was tested using a co-immunoprecipitation assay (Figure 3). COP1 attached to the GAL4 activation domain (GAD:COP1) and CO were made in an *in vitro* transcription/translation system and combined. GAD:COP1 was precipitated with anti-GAD antibody and CO was co-precipitated with GAD:COP1 (Figure 3). In contrast, CO was not co-immunoprecipitated with GAD alone.



**Figure 3** *In vitro* interaction between CO and COP1 detected by co-immunoprecipitation. <sup>35</sup>S-methionine-labeled CO, COΔB-box or COΔCCT was incubated with <sup>35</sup>S-methionine-labeled GAD:COP1 or GAD and co-immunoprecipitated with anti-GAD antibodies. Supernatant fractions and pellet fractions were resolved by SDS-PAGE and visualized by autoradiography using a phosphorimager. Quantification of the fractions of prey proteins that were co-immunoprecipitated by the indicated bait proteins GAD:COP1 or GAD. Error bars denote the standard error of the mean of two replicate experiments.

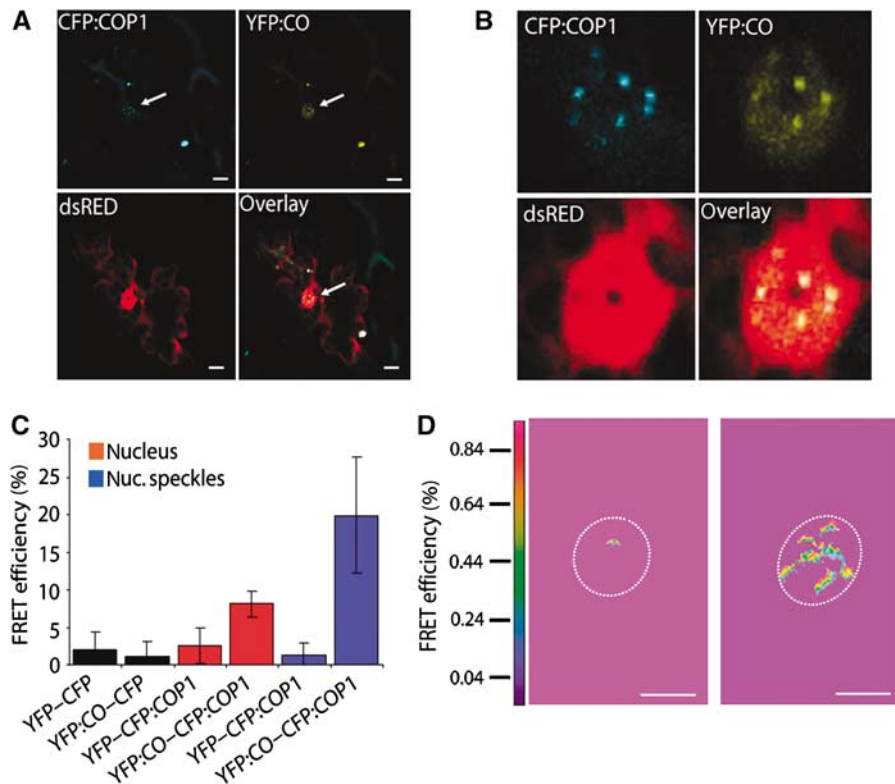
These experiments suggest that CO interacts with COP1 in the GAD:COP1 fusion protein. Fragments of CO were also combined with GAD:COP1 to determine which regions of CO are required for the interaction with COP1. Two segments of CO were tested: one containing the region between amino acids 107 and 373, which was called COΔB-box because it did not contain the zinc-finger B-boxes found at the N terminus of CO, and a second containing the region between amino acids 1 and 331, which was named COΔCCT, because the CCT domain near the C terminus of CO was removed. *In vitro* precipitation experiments demonstrated that COΔB-box was co-immunoprecipitated with GAD:COP1, whereas COΔCCT was not. Therefore, the N-terminal region containing the B-boxes is not required for interaction with COP1, suggesting that the interaction with COP1 is mediated by the C-terminal region of CO that contains the CCT domain.

Whether the interaction between CO and COP1 also occurred *in vivo* in plant cells was tested using fluorescent resonance energy transfer (FRET). Microprojectile bombardment was used to co-express cyan fluorescent protein (CFP):COP1 and yellow fluorescent protein (YFP):CO in leaf epidermal cells of *Arabidopsis*. CFP:COP1 and YFP:CO colocalized to the nucleus and also colocalized in speckles within the nucleus (Figure 4A and B). Physical interaction of CFP:COP1 and YFP:CO was tested by measuring FRET using photoacceptor bleaching, as previously described (Wenkel *et al*, 2006) (Figure 4C and D). Quantification of FRET signals demonstrated that FRET occurred between YFP:CO and CFP:COP1 both in the nucleus and specifically in nuclear speckles (Figure 4C and D). In control experiments using YFP and CFP, YFP:CO and CFP or YFP and CFP:COP1 FRET was detected at significantly lower levels (Figure 4C). These experiments demonstrate that YFP:CO and CFP:COP1 colocalize and physically interact in the nuclei of plant cells.

### COP1 and phyB have complementary roles in repressing CO protein levels under LDs and SDs

The genetic and molecular experiments described earlier supported the hypothesis that COP1 negatively regulates CO activity at the post-transcriptional level. Therefore, we tested the effect of COP1 on CO protein levels. First, CO protein abundance was examined in nuclei of WT Columbia, *co-10*, transgenic *35S:CO* and *cop1-4* plants harvested 16 h after dawn under LDs, when CO protein is expected to be at highest abundance (Valverde *et al*, 2004) (Figure 5A). As shown previously, CO was clearly detectable in *35S:CO* transgenic plants that overexpress the protein, but was below the level of detection in nuclei of WT plants. However, in *cop1-4* mutants, CO was clearly detected, suggesting that in WT plants COP1 has a major function in reducing CO protein levels at this time. Strong support that the protein detected in *cop1-4* mutants was indeed CO protein came from the analysis of *cop1-4 co-10* double mutants, in which the protein detected in *cop1-4* mutants was no longer present (Figure 5A).

CO mRNA shows a diurnal rhythm in abundance in WT plants and in *cop1-4* mutants, therefore the diurnal pattern of CO protein abundance was tested under LDs and SDs in *cop1-4* mutants (Figure 5B and C). Under SDs of 8 h light, *cop1-4* mutants flower dramatically earlier than WT plants (Figure 1) and CO protein was present for most but not all of the diurnal cycle (Figure 5C). CO was strongly detected soon



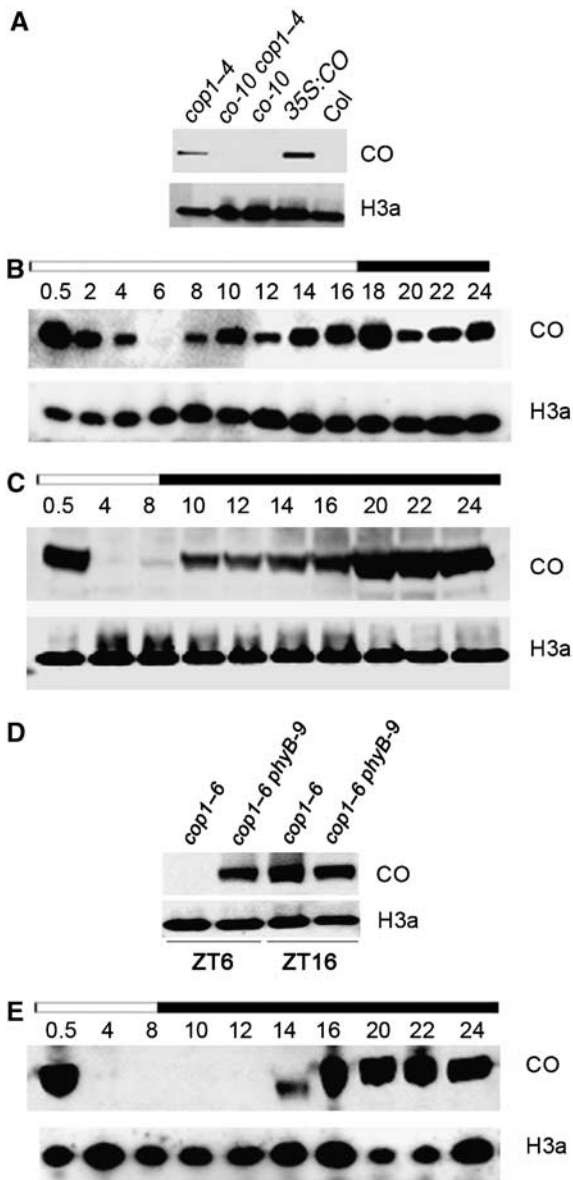
**Figure 4** CO protein physically interacts with COP1 in plant cells. (A) Transient co-expression of *35S:YFP:CO* and *35S:CFP:COP1* constructs. A *35S:dsRED* construct was cotransformed to highlight the transformed cell. The arrows represent the nucleus in which CO and COP1 are colocalized. (B) Enlargement of the nucleus shown in each of the panels represented in (A). (C) Quantification of FRET *in vivo* between CFP:CO and YFP:COP1. YFP:CO detected as an increase in CFP fluorescence after photobleaching of YFP. Quantification of FRET efficiencies after acceptor photobleaching measured in nuclei and nuclear speckles. Data are mean  $\pm$  s.d. of 10–20 cells from three separate experiments. (D) Visualization of increase in CFP fluorescence after YFP photobleaching. Left-hand panel, cells expressing CFP:COP1 and YFP, which exerts an effect as a negative control. Right-hand panel, cells expressing CFP:COP1 and YFP:CO. Scale bar: 6  $\mu$ m in (A) and 8  $\mu$ m in (D).

after dawn, was absent or present at much lower abundance 4 and 8 h after dawn, and then was present strongly for the remainder of the night from 10 to 24 h after dawn. The appearance of CO protein from 10 h after dawn is likely due to an increase in CO mRNA levels, as the abundance of CO mRNA increased steeply between 4 and 14 h after dawn in the same plants used for the protein analysis (Supplementary Figure 2). In contrast, CO mRNA abundance fell between 14 and 24 h after dawn, whereas CO protein levels were high throughout this time. This comparison suggests that impairing COP1 function causes CO protein to be relatively stable in the dark. However, the steep decline in CO protein abundance between 0.5 and 4 h after dawn suggests that a second post-translational mechanism, independent of COP1, might negatively regulate CO protein levels in the morning.

Under LDs of 16-h photoperiods, CO protein was detected from dawn until 4 h into the photoperiod, was undetectable 6 h after dawn and then was present for the remainder of the photoperiod and throughout the night (Figure 5B). This pattern was similar to that detected under SDs, but the protein was detectable for longer and was only absent at one time point, 6 h after dawn. The broader peak in CO protein under LDs is likely due to CO mRNA being expressed for longer under LDs, as previously described (Suarez-Lopez *et al*, 2001; Imaizumi *et al*, 2003).

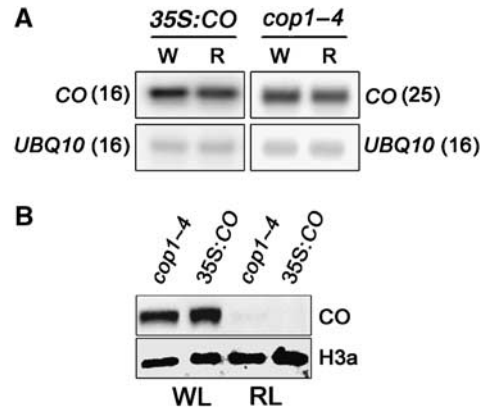
The photoreceptor phyB was previously shown to promote the degradation of CO protein early in the day in *35S:CO* plants, and this was proposed to contribute to the inhibitory

effect of phyB on flowering time (Valverde *et al*, 2004). To test whether phyB is responsible for the reduction in CO protein levels early in the day in *cop1* mutants, the *phyB-9 cop1-6* double mutant was tested for flowering time and CO protein levels. Under SDs, *phyB-9 cop1-6* plants flowered at a very similar time to *cop1-6* mutants, demonstrating that the early flowering of *cop1-6* mutants is not enhanced by loss of function of *phyB* (Supplementary Figure 3). Under LDs, the double mutant flowered significantly later than either single mutant, which indicates a complexity in the interaction between COP1 and phyB under these conditions that cannot be simply explained by regulation of CO protein levels (see Discussion). To test whether phyB is responsible for the reduction in CO protein early in the day in *cop1* mutants, protein was extracted from *phyB-9 cop1-6* and *cop1-6* plants 6 and 16 h after dawn under SDs. In the *cop1-6* plants, CO protein was undetectable 6 h after dawn, as observed for *cop1-4* mutants, but in *phyB-9 cop1-6* plants CO protein accumulated strongly 6 h after dawn (Figure 5D). In contrast, CO mRNA was present at similar levels in *cop1-6* and *phyB-9 cop1-6* plants 6 h after dawn (Supplementary Figure 3). These results indicate that phyB is required for post-transcriptional regulation of CO expression early in the day and independently of COP1. However, in the samples harvested 16 h after dawn CO protein was present at similar levels in *cop1-6* and *phyB-9 cop1-6* plants, indicating that phyB does not influence CO protein levels at that time of day (Supplementary Figure 3).



**Figure 5** Detection of CO protein in *cop1-4*, *cop1-6 phyB-9* and *spa1-7* plants. (A) CO protein was detected in *35S:CO* transgenic plants and *cop1-4* mutants, but not in WT Columbia, *co-10* or *cop1-4 co-10* mutants. Plants were grown under 16 h LDs and harvested 16 h after dawn. (B, C) CO protein in *cop1-4* mutants under 16 h LD or 8 h SD. Numbers above each lane represent hours after dawn that the sample was harvested. Light bar represents day; dark bar represents night. (D) CO protein detection in *cop1-6* and *cop1-6 phyB-9* plants grown under SDs. Samples were harvested 6 and 16 h after dawn. The reduction in CO protein at 6 h in *cop1-6* plants (see also (C)) does not occur in *cop1-6 phyB-9* plants. (E) CO protein detection in *spa1-7* mutants under 8 h SD. Numbers and bars as described for (B, C). In WT plants, CO protein could not be detected and therefore is not included as control ((A); Valverde *et al*, 2004). For all panels, histone 3a was used as a loading control.

SPA1 interacts with COP1 and is implicated in the degradation of some COP1 substrates. Recently, CO protein was shown to be more abundant in *spa1 spa3 spa4* triple mutants 16 h after dawn under LDs (Laubinger *et al*, 2006). The diurnal pattern of CO protein abundance in *spa1-7* mutant plants was tested to compare with that described for *cop1-4* mutants (Figure 5C and E). A similar pattern of CO protein



**Figure 6** Comparison of CO protein and mRNA in plants exposed to white or red light. *35S:CO* or *cop1-4* seedlings (12-day old) grown in LD were divided into two groups and exposed to 16 h red or white light, respectively. Samples were harvested for RNA and protein analysis at the end of the 16 h light period under both conditions. (A) CO and ubiquitin mRNA levels in *35S:CO* or *cop1-4* plants exposed to white (W) or red (R) light. Numbers in parentheses represent the numbers of cycles used to amplify the cDNA prior to separation on a gel. (B) CO and histone protein levels in the same plants used for (A). In both genotypes, CO is detected in white light (WL)-grown plants but not in red light (RL)-grown plants.

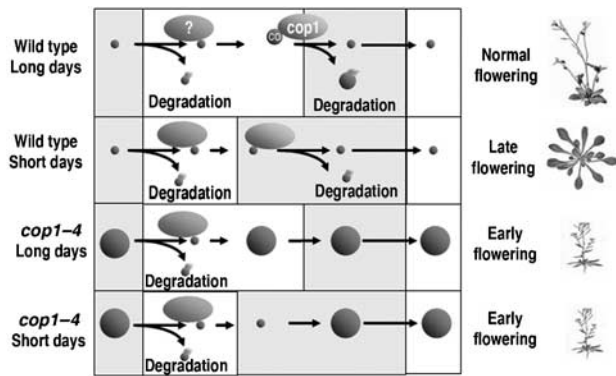
accumulation was observed in *spa1-7* and *cop1-4* mutants between 14 h after dawn and the following morning, but the rise in the abundance of the protein was delayed in the *spa1-7* mutant, so that it could not be detected until 14 h after dawn. In contrast, in the *cop1-4* mutant CO protein was strongly detected 10 h after dawn. These results suggest a functional relationship between COP1 and SPA1 proteins in the degradation of CO, and that of the four SPA proteins SPA1 has the major role in regulating CO levels. The delayed increase in CO abundance in *spa1-7* compared with *cop1-4* mutants might be due to the activity of other SPA proteins.

#### **Degradation of CO protein in red light is not impaired by the *cop1-4* mutation**

*Arabidopsis* plants flower later under red light and previously this was proposed to be at least partly due to degradation of CO protein under these conditions (Valverde *et al*, 2004). To test whether red light-mediated degradation of CO protein is also impaired in the *cop1-4* mutants, CO protein abundance was compared in *35S:CO* and *cop1-4* plants grown under white and red light (Figure 6). Similar levels of CO protein were detected in both lines grown under 16 h of white light (Figure 6). Furthermore, when both genotypes were exposed to 16 h of red light, CO protein abundance fell sharply in both *35S:CO* plants and *cop1-4* mutants (Figure 6). The reduced levels of CO protein observed in *cop1-4* mutants under red light compared with white light are not due to lower levels of CO mRNA, which were identical under both conditions (Figure 6). This result demonstrates that a COP1-independent mechanism is required for CO protein degradation under red light.

#### **Discussion**

We demonstrated that COP1 ubiquitin ligase is required to shape the diurnal pattern of CO protein accumulation as part



**Figure 7** Model for regulation of CO stability during photoperiodic flowering control in wild-type plants and *cop1-4* mutants. Photoperiodic flowering in *Arabidopsis* involves two mechanisms of CO protein degradation: a phyB-dependent mechanism occurs early in the day or in response to red light and a second mechanism involving COP1 occurs late in the day and during the night. (top) In wild-type plants under LDs, CO accumulates in the evening due to an increase in CO mRNA and photoreceptor-mediated repression of COP1. CO can promote FT expression at this time and thereby flowering. During the night, COP1 is active and causes rapid degradation of CO protein by ubiquitination and activity of the proteasome. (second from top) Under SD, CO mRNA is expressed during the night and the protein is degraded through COP1 activity. CO protein does not accumulate and FT mRNA is absent, resulting in late flowering. (second from bottom) In *cop1-4* mutants under LDs, CO is not degraded in the dark and accumulates to high levels. CO also accumulates to high levels at the end of the day, consistent with COP1 targeting CO for degradation at that time. However, CO protein still disappears early in the day, suggesting a COP1-independent mechanism of degradation at that time. (bottom) Under SDs in *cop1-4* mutants, CO accumulates to a high level during the night and promotes FT expression at higher level than in wild-type plants. Enhanced CO activity at these times is responsible for the early flowering of *cop1-4* mutants under SDs. In the morning, CO protein is degraded by a COP1-independent mechanism. The symbols represent CO protein abundance (red circles), COP1 (blue spheres), ubiquitin (small yellow circles on CO) and an unknown red light-activated degradation mechanism that is also active in the morning (dark orange spheres). A full-colour version of this figure is available at *The EMBO Journal* Online.

of the flowering response of *Arabidopsis* to photoperiod. COP1 delays flowering of WT plants under SDs by preventing CO protein accumulation during the night and thereby ensuring that FT transcriptional activation does not occur. Under LDs WT plants flower early, but even under these conditions COP1 modulates CO protein levels, lowering the abundance of the protein towards the end of the day and during the night. These effects on CO are consistent with the extreme early flowering of *cop1-4* mutants under LDs and SDs, the largely day length-insensitive phenotype of *cop1-4* mutants and suppression of these phenotypes to a large extent by *co* mutations. However, in *cop1-4* mutants CO protein abundance is still reduced in the early morning and in red light, indicating that a second mechanism independent of COP1 regulates CO protein abundance under these conditions. The mechanism that exerts an effect in the early morning is shown to depend on the phyB photoreceptor. Our observations place COP1 within the regulatory network for photoperiod perception and regulation of flowering in *Arabidopsis* (Figure 7), and extend the characterized functions of COP1 beyond those previously described in seedling photomorphogenesis.

### **COP1 reduces CO protein abundance to confer a photoperiodic flowering response**

COP1 represses seedling photomorphogenesis by catalysing the ubiquitination and therefore degradation of proteins that promote seedling photomorphogenesis (Osterlund *et al*, 2000; Seo *et al*, 2003; Duek *et al*, 2004; Jang *et al*, 2005; Yang *et al*, 2005). In addition to their effects on seedling development, *cop1* mutations severely impair the development of adult plants, although the mechanisms by which this occurs are less well understood. Altered adult traits include photoperiodic flowering so that *cop1* mutants flower at similar times under LDs and SDs (Mcnellis *et al*, 1994). In addition, *cop1* mutants flower in constant darkness in the presence of sucrose, whereas WT seedlings do not (Nakagawa and Komeda, 2004). Under these conditions, *cop1* mutants exhibit higher expression of FT and SOCI mRNAs than WT plants. We showed that the early flowering of *cop1-4* mutants under LDs or SDs largely depends on CO function and that in these mutants CO protein persists in the dark under SDs and LDs. These results suggest that post-translational regulation of CO is impaired in *cop1-4* mutants.

Under LDs, CO protein levels are high in *cop1-4* mutants throughout most of the day and rise earlier after dawn than under SDs, as shown for CO mRNA (Suarez-Lopez *et al*, 2001; Imaizumi *et al*, 2003). This effect on CO mRNA levels is at least in part due to FKF1- and GI-mediated degradation of the transcriptional repressor CYCLING DOF FACTOR 1 (CDF1), which is triggered by light, allowing CO mRNA abundance to rise earlier during the day under LDs (Imaizumi *et al*, 2005; Sawa *et al*, 2007). Perhaps surprisingly, our results indicate that under LDs, when CO promotes flowering of WT plants, COP1 also has a strong negative effect on CO protein levels. At the end of the day and during the night, CO protein accumulates to much higher levels in *cop1-4* mutants than WT plants, although CO mRNA abundance is actually lower than in WT plants. These results indicate that under LDs COP1 has an unexplained role in increasing CO mRNA abundance, and a major function in lowering CO activity by reducing CO protein abundance.

Under SDs, CO mRNA is expressed and rises during the night. Our demonstration of COP1-mediated degradation of CO protein in the dark under SDs provides a molecular explanation for why CO does not promote flowering under these conditions, and is consistent with previous demonstrations that application of proteasome inhibitors led to stabilization of the CO protein. The importance of this process in conferring a photoperiodic response is illustrated by the extreme early flowering of *cop1-4* mutants under SDs, which is responsible for almost abolishing the response to photoperiod. COP1 therefore have an important function in turning over CO protein in the light and dark under these conditions.

COP1 activity is higher in the dark than light. One mechanism by which this light regulation occurs is through exclusion of COP1 from the nucleus in the light (Von Arnim and Deng, 1994), whereas in addition COP1 activity is repressed by direct interaction with activated cryptochromes (Wang *et al*, 2001; Yang *et al*, 2001). Our observation that CO protein levels are very high in the dark under LD or SD in *cop1-4* mutants is consistent with COP1 activity being high in the dark under both day lengths and rapidly turning over CO protein. The increase in CO protein at the end of the day under LD

indicates that even in the light COP1 contributes to keeping CO protein levels low. COP1 activity might only be reduced and not fully suppressed at the light intensities used in these experiments. If so, then higher light intensities might promote flowering at least partly by more effectively repressing COP1 activity and allowing CO protein levels to rise higher. This would suggest a role for COP1 and CO in the regulation of flowering by light intensity.

#### **Degradation of CO protein in the morning or in red light does not require COP1**

Previously, two distinct post-transcriptional mechanisms were postulated to shape the diurnal pattern of CO protein accumulation. One of these occurred early in the day and involved a phyB-mediated signal and another occurred in the dark during the night (Valverde *et al*, 2004). Degradation of CO protein in red light was proposed to involve the same phyB pathway that caused rapid turnover of the protein early in the day. We observed that in *cop1-4* mutants the CO protein was still effectively degraded in red light and that there was a strong diurnal trough in CO protein levels early in the day. Degradation of CO early in the day was shown to require phyB but not COP1. The degradation of CO in red light likely occurs by the same phyB-dependent mechanism acting early in the day, as was shown by Valverde *et al* (2004), and this could be tested by comparing *cop1-6 phyB-9* and *cop1-6* plants under red light. Also, we cannot exclude that other phytochromes related to phyB may also have a function in CO regulation. In particular, phyC and phyE were demonstrated to influence flowering time (Halliday and Whitelam, 2003; Monte *et al*, 2003; Balasubramanian *et al*, 2006). Nevertheless, our data suggest that a second ubiquitin ligase may be responsible for phyB-mediated turnover of CO early in the day and in continuous red light (Figure 7). Interestingly, the bHLH transcription factor PHYTOCHROME INTERACTING FACTOR 3, which is involved in seedling photomorphogenesis and phytochrome signalling, was also recently shown to be degraded by a red light-activated ubiquitin-mediated process soon after dawn (Al-Sady *et al*, 2006). There might be a common mechanism promoting the degradation at dawn of several transcription factors involved in light signalling. Alternatively, a set of ubiquitin ligases might exist that specifically promote the degradation of individual transcription factors at this time. Further genetic and biochemical approaches are required to understand the mechanisms underlying CO protein degradation at dawn.

#### **Spatial regulation of photoperiodic response by COP1**

CO and *FT* are expressed in the vascular tissue and their expression in the phloem companion cells is sufficient to promote flowering (Takada and Goto, 2003; An *et al*, 2004). Furthermore, reducing *FT* expression specifically in the phloem companion cells delays flowering (Mathieu *et al*, 2007). Thus, the perception of photoperiod that is mediated through transcriptional and post-transcriptional regulation of CO likely takes place in the companion cells. Similarly, Cry2, which positively regulates CO accumulation, exerts an effect in the companion cells to regulate flowering (Endo *et al*, 2007). In contrast, phyB, a photoreceptor that delays flowering at least in part by reducing CO abundance, appears to exert an effect non-cell autonomously from the mesophyll cells (Endo *et al*, 2005). This result suggests that a signalling

step downstream of phyB exerts an effect non-cell autonomously to trigger degradation of CO protein, although definitive conclusions on the site of action of phytochromes influencing flowering will require a better understanding of the spatial requirement for other phytochromes, such as phyC and phyE. We showed that expression of *COP1* in the vascular tissue from the *SUC2* promoter complemented the flowering-time phenotype of *cop1-4* mutants under LDs and reduced *FT* mRNA levels. Under SDs, *cop1-4 SUC2:COP1* plants still flowered earlier than WT, but this was probably due to a CO-independent process causing early flowering in the *cop1-4* mutant, because *cop1-4 co-10* plants also flowered earlier than WT under SDs. In WT plants, *SUC2:COP1* also delayed flowering under LDs but not under SDs. The day length specificity of this effect suggests that the overexpression in companion cells affects flowering through CO, and indicates that COP1 levels are a limiting factor on CO degradation under these conditions. Taken together, these results suggest that COP1 exerts an effect in companion cells to regulate *FT* expression. This observation is consistent with our suggestion that COP1 exerts an effect to degrade CO at the end of the day and during the night, but not as part of the phyB pathway, which exerts an effect mainly in the morning or in red light. The temporal patterns of COP1 activity, therefore, support our understanding of the spatial pattern of activity of the pathways responsible for post-translational regulation of CO (Figure 7).

#### **COP1 and CO interact in vitro and in nuclear speckles in vivo**

COP1 directly interacts with target proteins and directs them for degradation (Hoecker, 2005; Jiao *et al*, 2007). CO is composed of three domains, zinc-finger B-boxes, a central domain and the C-terminal CCT domain (Wenkel *et al*, 2006). CO and COP1 interact directly *in vitro* as demonstrated by immunoprecipitation experiments. This interaction was almost abolished when the C-terminal part of CO was removed, suggesting that COP1 interacts with the C-terminal region of CO, as was previously observed for interactions between COP1 and COL3 or between CO and SPA1 (Datta *et al*, 2006; Laubinger *et al*, 2006). The interaction between COP1 and HY5 occurs through a defined domain that includes adjacent valine and proline residues that are essential for the interaction (Holm *et al*, 2001). Conserved pairs of valine-proline residues in the CCT domain of COL3 are also important for the interaction with COP1 (Datta *et al*, 2006). The region of CO that interacts with COP1 contains three VP motifs, but changing all of these to AA did not impair the interaction with COP1 *in vitro* (data not shown). A similar result was previously observed for the interaction between CO and SPA1 (Laubinger *et al*, 2006). Therefore, the interaction between CO and COP1 likely involves a different motif than observed for the interactions between COP1 and COL3 or HY5.

Direct interaction between COP1 and CO was further supported by transient expression of COP1 and CO fused to fluorescent proteins in *Arabidopsis* leaf cells. The proteins colocalized in the nucleus and both occurred in speckles. Previously, COP1 was shown to colocalize with its target proteins HY5, HYH and LAF1 in nuclear speckles in onion epidermal cells (Osterlund *et al*, 2000; Holm *et al*, 2002; Seo *et al*, 2003). COP1 speckles were proposed to represent

nuclear sites for proteasome-mediated protein degradation (Al-Sady *et al*, 2006). The presence of CO and COP1 in nuclear speckles similar to those observed for other targets of COP1-mediated degradation supports the idea that direct interaction between COP1 and CO is required for CO degradation in the nucleus. This degradation presumably requires the SPA proteins, perhaps acting directly in a larger order complex with COP1, as SPA proteins also regulate CO abundance at least at the end of a LD and interact directly with COP1 (Laubinger *et al*, 2006). The mechanism by which CO is degraded by the SPA-COP1 complex has therefore strong parallels with that of HY5. However, the precise relationship between SPA1 and COP1 activity and whether the proteins exert an effect in a larger order complex that interacts directly with substrates is still not clear (Saijo *et al*, 2003; Seo *et al*, 2003; Hoecker, 2005).

### **COP1 and the external coincidence model controlling flowering of *Arabidopsis* in response to photoperiod**

CO promotes flowering and *FT* transcription under LDs but not SDs. CO activity is proposed to be restricted to LDs by an external coincidence model in which circadian clock control and light signalling combine to trigger CO activity (Searle and Coupland, 2004; Imaizumi and Kay, 2006). The *cop1-4* mutant causes CO mRNA to accumulate earlier under SDs, so that *cop1-4* mutations may in part accelerate flowering under SDs by causing CO mRNA to be expressed in the light, as previously shown for *toc1-1* mutants (Yanovsky and Kay, 2002). However, the major time of expression of *FT* under SDs in *cop1-4* is later during the night, suggesting that the earlier phase of CO expression in *cop1-4* has a small part in the acceleration of flowering under these conditions. Rather the major role of COP1 in this system appears to be to degrade CO protein in the dark, and thereby ensure that no *FT* transcription occurs under SDs (Figure 7). The importance of this activity is demonstrated by the high abundance of CO protein in the *cop1-4* mutant under SDs and the extreme early flowering of *cop1-4* mutants under these conditions. During the final revision of this paper, another study described the role of cryptochrome signalling in suppressing COP1-mediated degradation of CO in the dark (Liu *et al*, 2008). Our data extend the model of photoperiodic flowering in *Arabidopsis* by providing a molecular explanation for why CO mRNA expression during the night in SDs does not lead to *FT* transcription and promotion of flowering. The day length-insensitive early-flowering phenotype of *cop1-4* mutants and the strong suppression of this phenotype caused by *co* null alleles demonstrate that degradation of CO under SDs is essential in conferring a photoperiodic flowering response.

## **Materials and methods**

### **Plant material**

WT *Arabidopsis thaliana* plants and all mutants used in this study were Col-0. The *cop1-4* allele was previously characterized (McNellis *et al*, 1994). The *co-10* allele was previously used (Laubinger *et al*, 2006) and was confirmed to have a T-DNA insertion 342 bp after the ATG. Homozygous *cop1-4 co* mutant plants were found using PCR-based markers. The *cop1-6 phyB-9* and *cop1-6* seeds were kindly provided by Dr Jorge Casal (Boccalandro *et al*, 2004).

### **Analysis of flowering time**

For analysis of flowering time and gene expression, plants were grown on soil in controlled environment rooms under LDs (16 h light–8 h dark) or SDs (8 h light–16 h dark). Flowering time was measured by scoring the number of rosette and cauline leaves on the main stem of at least eight individuals. Data are expressed as average  $\pm$  s.d.

### **mRNA expression analysis**

*Arabidopsis* RNA was isolated with the Plant RNeasy kit (Qiagen) according to the manufacturer's instructions. RNA was analysed by RT-PCR. Detailed protocols and the origins of the primer sequences are presented in Supplementary data.

### **Plant transformation**

The *COP1* full-length cDNA was isolated by RT-PCR and produced as entry clone through BP reaction of Gateway system from Invitrogen. Then, the entry clone was utilized for the construction of destination vectors for plant transformation, FRET experiments and *in vitro*-binding assay. All plasmids for plant transformation were introduced into *Agrobacterium* strain GV3101 (pMP90RK) and transformed into WT Columbia, *cop1-4* or *SUC2:CO* (An *et al*, 2004) plants by the floral dip method (Clough and Bent, 1998).

### **In vitro-binding assay**

For the *in vitro* expression of GAD:COP1, we produced the vector pJIC39 containing pT7:GAD:GATEWAY cassette and T7 terminator, so that by LR reaction with the COP1 entry clone, the construct expressing GAD::COP1 is produced. Vector (Wenkel *et al*, 2006; Turck *et al*, 2007) pJIC26 is similar but contains only the GAD domain and was used for expressing full open reading frame of CO or parts of the ORF. CO $\Delta$ B-box and CO $\Delta$ CCT (Laubinger *et al*, 2006) were also tested for the binding with COP1. The detailed method used for the *in vitro* precipitation experiments is presented in Supplementary data.

### **Confocal microscopy, CO:COP1 colocalization and FRET analysis**

To express CFP:COP1 and YFP:CO in plants, the CO and COP1 genes were cloned into the GATEWAY vectors pENSG:CFP or pENSG:YFP by recombination reaction. In these vectors, CFP:COP1 and YFP:CO are expressed under the control of the constitutive 35S promoter (Laubinger *et al*, 2006). The method used to analyse FRET is described in detail in Supplementary data.

### **Immunological techniques**

WT Columbia, *cop1-4* and *spa1-7* were grown in temperature-controlled light cabinets either under LDs (16 h light and 8 h dark) or SDs (8 h light and 16 h dark). Plants were grown on solid germination medium for 2 weeks, harvested at specified zeitgeber time (ZT), frozen in liquid nitrogen and kept at  $-80^{\circ}\text{C}$  until further use. For the red light experiments, 35S:CO and *cop1-4* plants were grown in LD (16 h light–8 h dark) for 12 days, moved to red light conditions at ZT 0 and maintained for 16 h under red light. Nuclear extracts were prepared from the plants at different ZT times as described previously (Valverde *et al*, 2004). Nuclear proteins (17  $\mu\text{g}$ ) were separated employing 10% bis-Tris NuPAGE gels (Invitrogen), transferred to nitrocellulose membranes and probed with an anti-CO antibody followed by a horseradish peroxidase-conjugated secondary antibody. Immunoreactive proteins were visualized by Pico chemiluminescence substrate system (Pierce). The membrane was subsequently reprobed with an antibody against histone H3a (Abcam) as a loading control.

### **Supplementary data**

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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