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Phosphorylation of CONSTANS and its COP1-dependent degradation during photoperiodic flowering of Arabidopsis

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SUMMARY

Seasonal flowering involves responses to changes in day length. In *Arabidopsis thaliana*, the CONSTANS (CO) transcription factor promotes flowering in the long days of spring and summer. Late flowering in short days is due to instability of CO, which is efficiently ubiquitinated in the dark by the CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) E3 ligase complex. Here we show that CO is also phosphorylated. Phosphorylated and unphosphorylated forms are detected throughout the diurnal cycle but their ratio varies, with the relative abundance of the phosphorylated form being higher in the light and lower in the dark. These changes in relative abundance require COP1, because in the *cop1* mutant the phosphorylated form is always more abundant. Inactivation of the PHYTOCHROME A (PHYA), CRYPTOCHROME 1 (CRY1) and CRYPTOCHROME 2 (CRY2) photoreceptors in the *phyA cry1 cry2* triple mutant most strongly reduces the amount of the phosphorylated form so that unphosphorylated CO is more abundant. This effect is caused by increased COP1 activity, as it is overcome by introduction of the *cop1* mutation in the *cop1 phyA cry1 cry2* quadruple mutant. Degradation of CO is also triggered in red light, and as in darkness this increases the relative abundance of unphosphorylated CO. Finally, a fusion protein containing truncated CO protein including only the carboxy-terminal region was phosphorylated in transgenic plants, locating at least one site of phosphorylation in this region. We propose that CO phosphorylation contributes to the photoperiodic flowering response by enhancing the rate of CO turnover via activity of the COP1 ubiquitin ligase.

Keywords: photoperiodic flowering, phosphorylation, ubiquitination, phytochrome, *Arabidopsis thaliana*.

INTRODUCTION

Flowering marks the transition from the vegetative to the reproductive phase in plants and is often controlled by responses to seasonal cues such as day length or winter cold (Andres and Coupland, 2012). These adaptive responses ensure that flowering is timed to maximize reproductive success. The capacity of plants to perceive and respond to changes in day length was first recognized almost a century ago and called photoperiodism (Garner and Allard, 1923). *Arabidopsis thaliana* is a genetic model system for photoperiodic responses in plants, and flowers earlier when exposed to long days (LDs) with 16 h of light than to short days (SDs) with 10 or 8 h of light (Redei, 1962). Mutations that impair photoperiodic responses were identified by screening for plants that flowered later than the wild type under LDs, but that were unaffected in their flowering time under SDs (Redei, 1962; Koornneef *et al.*, 1991). These mutants defined a genetic pathway called the long-day or photoperiodic flowering pathway. The transcription factor (TF) CONSTANS (CO) plays a central role in this pathway and its abundance is higher under LDs, when it promotes flowering. Here we show that CO protein is phosphorylated and that this plays a role in regulating the abundance of the protein, as the phosphorylated form is preferentially degraded when the CONSTITUTIVE

PHOTOMORPHOGENIC 1 (COP1) ubiquitin ligase complex is active. Furthermore, partially due to photoreceptor-mediated inhibition of the activity of COP1, the relative abundance of phosphorylated CO is increased in the light under LDs when CO promotes flowering.

CONSTANS is a member of the B-box zinc finger family (Khanna *et al.*, 2009). It contains two B-boxes near the N-terminus that are functionally important, as several mutant alleles cause non-synonymous changes in these domains (Putterill *et al.*, 1995; Robson *et al.*, 2001). In addition, at the C-terminus the protein contains a CONSTANS, CONSTANS-LIKE, TIMING OF CAB1 (CCT) domain that mediates DNA binding and is functionally important based on analysis of mutant alleles (Strayer *et al.*, 2000; Robson *et al.*, 2001). CONSTANS binds directly to motifs in the proximal promoter of its major target gene *FLOWERING LOCUS T* (*FT*) (Tiwari *et al.*, 2010; Song *et al.*, 2012). These motifs are related to those recognized by other CCT domain proteins and are required for transcription of *FT* in LDs (Adrian *et al.*, 2010; Gendron *et al.*, 2012). Transcription of *CO* occurs in the vascular tissue and its mis-expression from phloem-specific promoters, such as that of the *SUCROSE TRANSPORTER 2* (*SUC2*) gene, is sufficient to promote flowering and complement the *co* mutation (Takada and Goto, 2003; An *et al.*, 2004). *FT* is also expressed in the vascular tissue in a CO-dependent manner (Takada and Goto, 2003; Adrian *et al.*, 2010). Furthermore, over-expression of CO from the cauliflower mosaic virus 35S promoter, which is active in a broad range of cell types, only allows increased transcription of *FT* in the vascular tissue, suggesting that CO activity is restricted to these cells at the post-transcriptional level or that *FT* transcription is prevented in other cell types, perhaps by the structure of the chromatin at the locus (Adrian *et al.*, 2010). After transcriptional activation by CO, FT protein moves to the shoot apex (Corbesier *et al.*, 2007; Jaeger and Wigge, 2007; Mathieu *et al.*, 2007), where it induces transcriptional reprogramming of the meristem to form an inflorescence meristem and subsequently flowers (Schmid *et al.*, 2003; Torti *et al.*, 2012).

In addition to its spatial regulation, *CO* expression is controlled by day length so that it only promotes *FT* transcription under LDs. At one level this occurs through transcriptional regulation of *CO*. Transcription of the gene is controlled by the circadian clock with a peak in mRNA abundance under LDs late in the light period and during darkness (Suarez-Lopez *et al.*, 2001). The coincidence between a peak in the mRNA abundance and exposure of plants to light occurs only under LDs, whereas under SDs a high-amplitude peak in *CO* mRNA occurs exclusively in the dark. The amplitude of the peak in mRNA abundance when plants are exposed to light under LDs is increased by the FLAVIN KELCH REPEAT F BOX 1 (FKF1) ubiquitin ligase (Imaizumi *et al.*, 2005), which is activated by light through its attached flavin chromophore leading to degradation of the CYCLING DOF TFs that are direct repressors of *CO* transcription (Imaizumi *et al.*, 2005; Sawa *et al.*, 2007; Fornara *et al.*, 2009). The removal of these repressors boosts *CO* transcription in the light under LDs.

Post-translational modification is also important in the regulation of CO activity by day length (Valverde *et al.*, 2004). In the dark, CO is degraded by the 26S proteasome following ubiquitination by the COP1 (Jang *et al.*, 2008; Liu *et al.*, 2008) and SUPPRESSOR OF PHYA1 (SPA1) (Laubinger *et al.*, 2006b; Jang *et al.*, 2008; Liu *et al.*, 2008) ubiquitin ligase complex. By contrast, on exposure of plants to light, activity of the COP1–SPA1 complex is reduced by direct binding of CRY1 and CRY2 photoreceptors to SPA1 in a blue (B) light-dependent manner (Lian *et al.*, 2011; Liu *et al.*, 2011; Zuo *et al.*, 2011). PHYA also enhances the accumulation of CO in the afternoon of LDs in response to far-red (FR) light (Valverde *et al.*, 2004), by suppressing COP1–SPA1 activity (Sheerin *et al.*, 2015). In addition to light-mediated inhibition of COP1–SPA1, direct interaction between CO and the ubiquitin ligase FKF1 increases CO levels (Song *et al.*, 2012). Direct interaction between these proteins stabilizes CO in the light under LDs by an unknown mechanism (Song *et al.*, 2012). Thus post-translational regulation refines the duration and amplitude of the peak in CO protein under LDs independently of its transcriptional regulation. In addition to these events that occur towards the end of a LD, PHYTOCHROME B (PHYB) functions early in the morning to suppress CO accumulation specifically under red (R) light conditions (Valverde *et al.*, 2004; Jang *et al.*, 2008). This reduction of CO levels by PHYB might be mediated by HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1 (HOS1), another E3 ubiquitin ligase that also targets CO for degradation via the proteasome under cold stress (Jung *et al.*, 2012; Lazaro *et al.*, 2012). Therefore, post-translational regulation of CO stability by different mechanisms plays a major role in conferring the photoperiodic response.

Phosphorylation is a further post-translational modification that influences the stability and activity of TFs (Hunter and Karin, 1992; Hill and Treisman, 1995; Sugiyama *et al.*, 2008). In plants, TFs that contribute to photoreceptor signalling pathways, the oscillator of the circadian clock and clock-controlled output pathways are regulated by phosphorylation (Sugano *et al.*, 1999; Hardtke *et al.*, 2000; Duek *et al.*, 2004; Fujiwara *et al.*, 2008; Bu *et al.*, 2011). The efficiency and rate of ubiquitination can be influenced by phosphorylation, and degradation of several TF substrates of COP1 is altered by phosphorylation (Hoecker, 2005). For example, the TFs LONG HYPOCOTYL 5 (HY5) and LONG HYPOCOTYL IN FAR RED 1 (HFR1) are phosphorylated, and this modification influences their rate of degradation as mediated by COP1 (Hardtke *et al.*, 2000; Duek *et al.*, 2004; Park *et al.*, 2008). The effect of phosphorylation on

the turnover of these COP1 substrates differs; during the night, the unphosphorylated HY5 protein is more efficiently degraded (Hardtke *et al.*, 2000), whereas at the same time of day the phosphorylated form of HFR1 is the less abundant (Duek *et al.*, 2004; Park *et al.*, 2008).

In this study, we explore whether CO is phosphorylated, and if so how this affects its regulation by photoreceptors and COP1. Western blot analysis of seedlings expressing *CO* from different promoters revealed two forms of CO protein, and we show that one of these represents a phosphorylated form. This phosphorylated form of CO is demonstrated to be preferentially degraded in the dark in a process dependent on COP1 ubiquitin ligase and also under R light. We propose that post-translational regulation by phosphorylation contributes to the instability of CO in the darkness and under SDs and thereby to the control of flowering by day length.

RESULTS

CONSTANS protein is phosphorylated

Two forms of CO protein that migrated differently after electrophoresis were detected on Western blots of nuclear protein extracts from *35S::CO* transgenic plants (Figure 1a). The *35S* promoter is active in most cell types whereas the *CO* promoter is mainly active in vascular tissue (An *et al.*, 2004). Therefore, to test whether the results obtained with *35S::CO* were due to ectopic expression in other cell types, CO protein was also examined in *SUC2::HA:CO* plants, in which CO is fused to the hemagglutinin (HA) epitope tag and is specifically expressed in the phloem companion cells, where it promotes flowering, and in *pCO::HA:CO* plants, where it is expressed under its native promoter. In all T_1 plants tested, the *pCO:HA:CO* transgene over-complemented the late-flowering phenotype of *co-10* mutants,

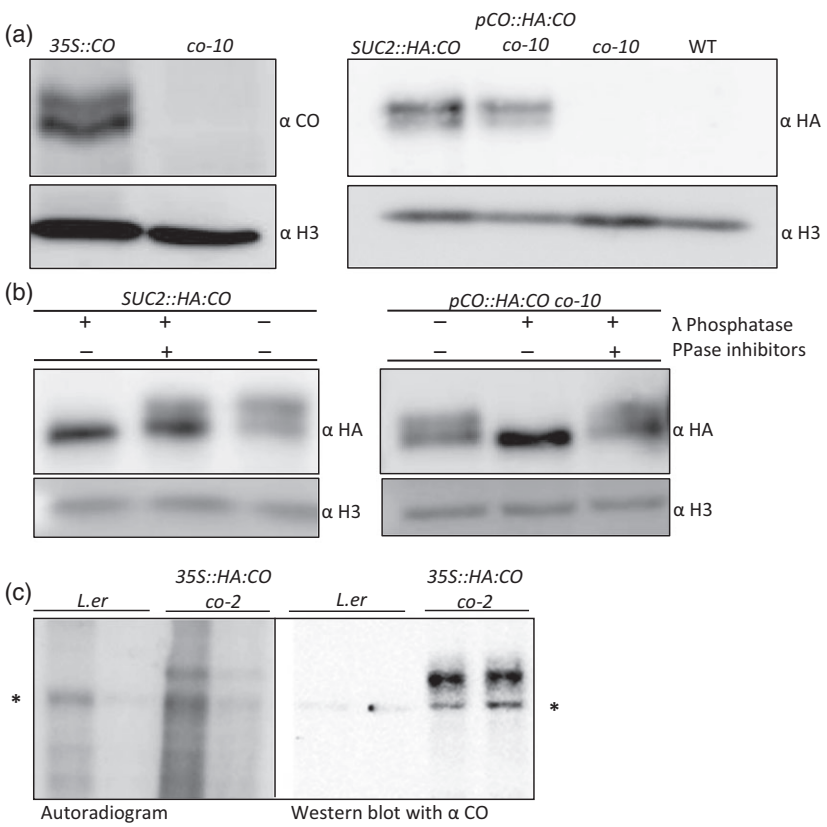

Figure 1. CONSTANS (CO) protein is phosphorylated.

(a) Two forms of nuclear CO were detected in transgenic Arabidopsis seedlings expressing CO under the constitutive *CaMV35S* promoter (*35S*), the phloem companion cell-specific promoter (*SUC2*) and CO native promoter (*pCO*). Seedlings were grown for 10 long days (LDs; 16-h light/8-h dark) before being harvested at Zeitgeber time (ZT) 16. Nuclear proteins were extracted and CO and HA:CO were detected using anti-CO and anti-HA antibodies, respectively. Neither form was detected in *co-10* or Columbia plants. Anti-H3 antibodies were used to detect H3 as loading control.

(b) The slower-migrating form of CO is susceptible to phosphatase treatment. Nuclear proteins were treated with lambda (λ) phosphatase (+) or mock (–) and phosphatase (PPase) inhibitors (+) or mock (–). Anti-HA antibodies were used to detect HA:CO protein and anti-H3 antibodies were used to detect H3 as a loading control.

(c) *In vivo* labelling of CO protein with radioactive phosphate. Nuclear proteins were extracted from Arabidopsis seedlings, treated for 3 days with gamma-labelled ATP and immunoprecipitated with anti-HA antibodies before being loaded on an SDS-PAGE gel. One part of the gel was exposed to a film (left panel) and the other part was transferred to a membrane and probed with anti-CO antibodies (right panel). The phosphorylated and non-phosphorylated forms are not separated in the right-hand panel due to the gel and transfer systems used to accelerate handling of radioactive gels (see Experimental Procedures). Asterisks mark non-specific bands that appeared in all the samples, including the controls.

causing earlier flowering than in the wild type under SDs (Figure S2). A representative transformant was selected based on flowering time and shown to exhibit diurnal expression patterns of *CO* and *FT* mRNAs (Figure S2). Western blotting revealed that both forms of CO are also present in *pCO:HA:CO* and *SUC2::HA:CO* plants, and that these forms are not therefore a result of ectopic expression in *35S:CO* plants (Figure 1a and Figure S1 in Supporting Information). These data demonstrate that both forms of CO protein are found in tissues where CO is active in promoting flowering and *FT* transcription.

To examine whether phosphorylation of CO contributes to these different forms, nuclear protein extracts were incubated with lambda phosphatase (Figure 1b). After incubation of protein extracts from either *SUC2:HA:CO* or *pCO:HA:CO co-10* with phosphatase the slower-migrating form was no longer detected. By contrast, this slower-migrating form was still detected after control incubations lacking the phosphatase or in which samples were treated simultaneously with phosphatase and phosphatase inhibitor (Figure 1b). These experiments suggested that CO protein is phosphorylated *in vivo* and that the phosphorylated form is represented by the slower-migrating band.

Further evidence for CO phosphorylation was obtained from *in vivo* labelling experiments using gamma-labelled ATP. Transgenic *35S:HA:CO co-2* plants and wild-type Landsberg *erecta* (L*er*) controls were grown under LDs for 12 days and transferred to MS medium containing 500 μCi gamma-labelled ATP for three LDs. Proteins were extracted and immunoprecipitated with anti-HA antibody. The immunoprecipitated proteins were then separated by electrophoresis and part of the gel was dried and exposed to a film whereas the other was transferred to a membrane and probed with CO antibody. Labelled phosphorylated protein that migrated at the same position as HA:CO detected on the Western blot was present in the transgenic plants but not in wild-type controls (Figure 1c). Taken together, the phosphatase treatment as well as the *in vivo* labelling approach support the idea that CO protein is phosphorylated *in vivo*.

Phosphorylated CO is preferentially degraded in the dark

The COP1 ubiquitin ligase is required for rapid turnover of CO in the dark (Jang *et al.*, 2008; Liu *et al.*, 2008). Therefore, the levels of the phosphorylated and unphosphorylated forms of HA:CO protein were tested in wild-type and *cop1-4* mutant plants growing under LDs (16-h light/8 dark) to determine whether one of the forms of CO is preferentially degraded in the dark. After 16 h in the light, CO protein accumulated in both *SUC2::HA:CO* and *SUC2::HA:CO cop1-4* plants and the phosphorylated form was noticeably more abundant than the unphosphorylated (Figure 2a). After 8 h in the dark, soon before dawn of the following day, abundance of HA:CO protein was much reduced in *SUC2::HA:CO* plants but was similarly abundant to dusk in the *SUC2::HA:CO cop1-4* plants, as expected (Figure 2a). In the HA:CO protein that was still detected in the dark in *SUC2::HA:CO* plants, the unphosphorylated form was more abundant, whereas in *SUC2::HA:CO cop1-4* the phosphorylated form was more abundant, as observed during light exposure (Figures 2a and S3). Quantification of these Western blots indicated that the ratio of phosphorylated to unphosphorylated CO forms was higher than 1.0 in both genotypes after 16 h of light, whereas in *SUC2::HA:CO* the ratio changed to favour the unphosphorylated form after 8 h in the dark. By contrast, in *SUC2::HA:CO cop1-4* the ratio remained approximately the same in the dark as before dusk (Figure 2b). This quantification supports the idea that the phosphorylated form of CO is more rapidly degraded in the dark when COP1 is active but not in the *cop1-4* mutant. Moreover, Western blot analysis of the diurnal regulation patterns of CO in both *pCO::HA:CO co-10* and *SUC2::HA:CO* plants grown under 16-h LDs detected sharp increases in the ratio of the phosphorylated form to the unphosphorylated form in the afternoon and rapid reversion of the ratio upon transfer to the dark (Figure 2c,d). For example, comparison of the 12 and 18-h time points illustrates the relative reduction in abundance of the phosphorylated form on transfer to the dark. Remarkably, in the *cop1-4* mutant background the phosphorylated form of CO remained more abundant than the unphosphorylated form throughout the light–dark cycle (Figure 2e). Taken together, these results indicate that COP1 is required for the fast degradation of phosphorylated CO protein in the dark.

Photoreceptor-mediated post-translational regulation of CO protein involves COP1

Photoreceptors increase levels of CO protein in the afternoon of LDs (Valverde *et al.*, 2004). To further characterize the relationship between COP1 and the photoreceptors in CO regulation, a quadruple *phyA cry1 cry2 cop1* mutant was generated by crossing the triple mutant *phyA cry1 cry2* to *cop1-4*. These quadruple mutant plants flowered as early as *cop1-4* under both LDs and SDs (Figure 3a), indicating that COP1 is epistatic to these photoreceptor mutations with respect to flowering time. To test whether this early flowering is due to CO activity, the diurnal expression patterns of *CO* and *FT* mRNA under LDs and SDs were analysed (Figure 3b). The abundance of *FT* mRNA, but not of *CO* mRNA, was strongly reduced in the *phyA cry1 cry2* triple mutant under both LDs and SDs (Figure 3b). However, in the *phyA cry1 cry2 cop1* quadruple mutant *FT* mRNA was strongly elevated to similar levels as in the *cop1-4* mutant (Figure 3b). Similar results were obtained in LDs and SDs, demonstrating that *cop1-4* is epistatic to the photoreceptor mutations in regulating *FT* transcription, and possibly CO protein activity. To further test for a corre-

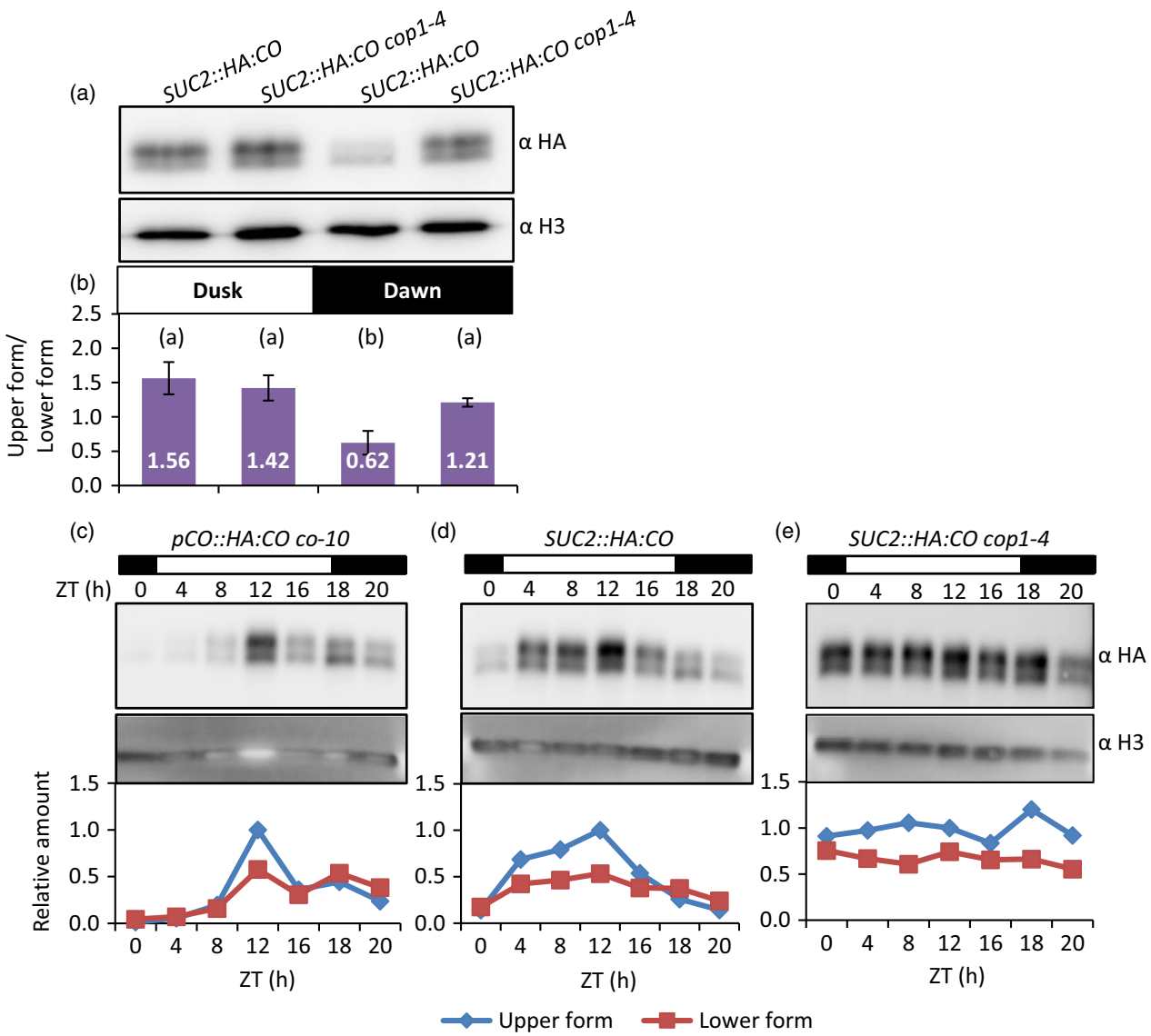

Figure 2. Phosphorylated CONSTANS (CO) is preferentially degraded by CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) in the dark.
(a) The HA:CO nuclear protein levels detected by Western blot at dusk (ZT 16) or dawn (ZT 24) of long days (LDs; 16-h light/8-h dark). Transgenic *SUC2::HA:CO* seedlings, grown under 16-h LDs, accumulate high levels of phosphorylated HA:CO at dusk but not at dawn. By contrast in *SUC2::HA:CO cop1-4* background phosphorylated HA:CO levels remain high at dawn. Anti-HA antibodies were used to detect HA:CO protein and anti-H3 antibodies were used to detect H3 as loading control.
(b) Ratio of phosphorylated (upper form) and non-phosphorylated (lower form) HA:CO in *cop1-4* or Col backgrounds at dusk or dawn of LDs. Western blots of three independent experiments were quantified using ImageJ. Signals for bands representing each form of HA:CO were divided by the signal for the H3 control before the ratio of the two forms was calculated. Numbers represent the mean and error bars indicate ± SD. Statistical differences between groups were determined with one-way analysis of variation (ANOVA) and multiple testing with the Holm–Sidak method ($\alpha \leq 0.05$).
(c)–(e) Diurnal regulation of the two forms of CO in different genotypes. The relative amount of each form of the protein detected on each Western blot is illustrated in the graphs. Western blots were quantified using ImageJ and signals for each form of HA:CO were divided by the signal for the phosphorylated form at ZT 12 of the same genotype to provide the values shown in the vertical axis. The horizontal axis is Zeitgeber time (ZT) represented as hours after dawn. White bars illustrate the duration of day, black bars the duration of night.

lation between photoreceptor activity and COP1 in the regulation of CO protein abundance, *SUC2::GSTAP:CO* was constructed and introduced into *phyA cry1 cry2 cop1*, *phyA cry1 cry2* and *co-10* mutants. The diurnal abundance of the two forms of CO in the different transgenic backgrounds was examined (Figure 3c–e). In the absence of the three photoreceptors, GSTAP:CO protein levels were reduced in the afternoon of LDs compared with *SUC2::GSTAP:CO co-10* (Figure 3c,d). Similar results were obtained with 35S::CO plants (Figure S4). Interestingly, the phosphorylated form of CO in the triple photoreceptor mutant background was greatly reduced and less abundant than the non-phosphorylated form, suggesting that the photoreceptors may act to promote CO phosphorylation as

observed for other TFs (Shen *et al.*, 2009; Kusakina and Dodd, 2012), or that hyperactivity of COP1 in those plants contributes to the strong reduction in levels of phosphorylated CO protein. We reasoned that if the photoreceptors promote CO phosphorylation then in the *phyA cry1 cry2 cop1* quadruple mutants the phosphorylated form should be present at lower levels than the unphosphorylated CO. However, in *SUC2::GSTAP:CO phyA cry1 cry2 cop1* plants both forms of CO strongly accumulated at all time points for which the protein was detected (Figure 3e). Thus the photoreceptors probably do not directly promote phosphorylation of CO, but rather enable the increase in phosphorylated CO indirectly by suppressing COP1 and thereby reducing degradation of the phosphorylated form. To verify that the increase of GSTAP:CO protein in the quadruple mutants compared with the triple photoreceptor mutants was due to post-translational regulation of the protein, levels of *CO* mRNA were quantified in the different transgenic plants (Figure S5). In contrast to protein levels, *CO* mRNA levels were lower in the *SUC2::GSTAP:CO phyA cry1 cry2 cop1* plants than in *SUC2::GSTAP:CO phyA cry1 cry2*. Taken together, these experiments support the idea that the photoreceptors increase the abundance of CO protein at the post-transcriptional level by repressing COP1 activity, and that this also increases the ratio of phosphorylated to unphosphorylated CO protein.

Effects of red or blue light on the relative abundance of the phosphorylated form of CO

The CO protein is degraded not only in the dark by COP1 but also under R-light conditions via a PHYB-activated pathway that acts early in the day to repress CO protein levels (Valverde *et al.*, 2004). To test which form of CO is preferentially degraded via this R-light-mediated pathway, the abundance of phosphorylated and unphosphorylated CO protein was examined throughout a 16-h LD of R or white (W) light in plants that express *HA:CO* from *SUC2* or the *CO* native promoters (Figure 4a,b). The overall levels of HA:CO protein were reduced compared with plants exposed to W light (Figure 4a,b). In particular, the abundance of the phosphorylated form was lower so that the unphosphorylated form generally appeared to be more abundant under R light. The relative abundance of the two forms under R light was examined in more detail for plants grown in both conditions for 12 h (Figure S6). The ratio of phosphorylated to unphosphorylated forms was >1.0 for plants grown under W light, but lower than 1.0 for those grown under R light (Figure S6). Thus, these results indicate that under R light as well, the preferred substrate for degradation is the phosphorylated form of CO. Exposure of plants to blue (B) light stabilizes CO protein (Valverde *et al.*, 2004), and on exposure of *pCO::HA:CO* plants to B light the ratio of phosphorylated to unphosphorylated HA:CO was >1.0 at 12 h after dawn (Figure S6c). Thus, as in W light, when CO protein is stabilized in response to B light the relative abundance of the phosphorylated form increases.

The C-terminal part of CO is sufficient to confer phosphorylation but not for dark degradation

The CO protein is composed of three major domains, an N-terminal zinc-finger B-box domain, a middle region and the C-terminal CCT domain (Putterill *et al.*, 1995; Strayer *et al.*, 2000; Robson *et al.*, 2001). The CCT domain-containing region of CO was previously reported to be important for the interaction with COP1 (Jang *et al.*, 2008) and SPA1 (Laubinger *et al.*, 2006b), because a truncated protein lacking this domain [1–331 amino acids (aa)] was unable to bind these proteins *in vitro*. Nevertheless, a short C-terminal version of CO (272–373 aa) was not sufficient to interact with SPA1 (Laubinger *et al.*, 2006b). To better understand the importance of this domain in the post-translational regulation of CO *in vivo*, a C-terminal region (297–373 aa) that includes the CCT domain (CO-CCT) was fused to yellow fluorescent protein (YFP) and expressed in wild-type Col plants from the *35S* promoter (Figure 5a). To evaluate whether this domain is sufficient for dark-mediated degradation *in planta* the accumulation of CO-CCT:YFP was compared with that of full-length CO (CO-FL:YFP) during a LD diurnal cycle (Figures 5b,c and S7). In contrast to CO-FL:YFP, which demonstrated a sharp reduction in abundance in the dark (Figure 5b), CO-CCT:YFP was present at similar levels in light and dark and no apparent degradation was detected (Figure 5c). These results imply that the C-terminal region including the CCT domain is not sufficient for degradation in the dark. However, Western blot analysis of CO-CCT:YFP revealed two forms of the protein, suggesting that it was phosphorylated (Figure 5c,d), and possible phosphorylation sites were present in this segment of the CO sequence (Figure 5e). To test whether these two forms indeed represent a phosphorylated form as for the full-length protein, a phosphatase treatment was performed on nuclear extracts of tobacco plants transiently expressing HA:CO-FL or HA:CO-CCT proteins (Figure 5f). Two forms of both proteins were detected in this transiently expressed protein. The upper forms of both HA:CO-FL and HA:CO-CCT extracted from *Nicotiana benthamiana* nuclei were susceptible to phosphatase treatment, indicating that the short fragment of CO containing the CCT domain is sufficient to confer phosphorylation. Taken together, these results suggest that the C-terminal part of CO contains at least part of the phosphorylated region, but that this is not sufficient for the degradation of CO in the dark.

DISCUSSION

Post-translational modification of CO protein is regulated by photoperiod and contributes to the flowering response

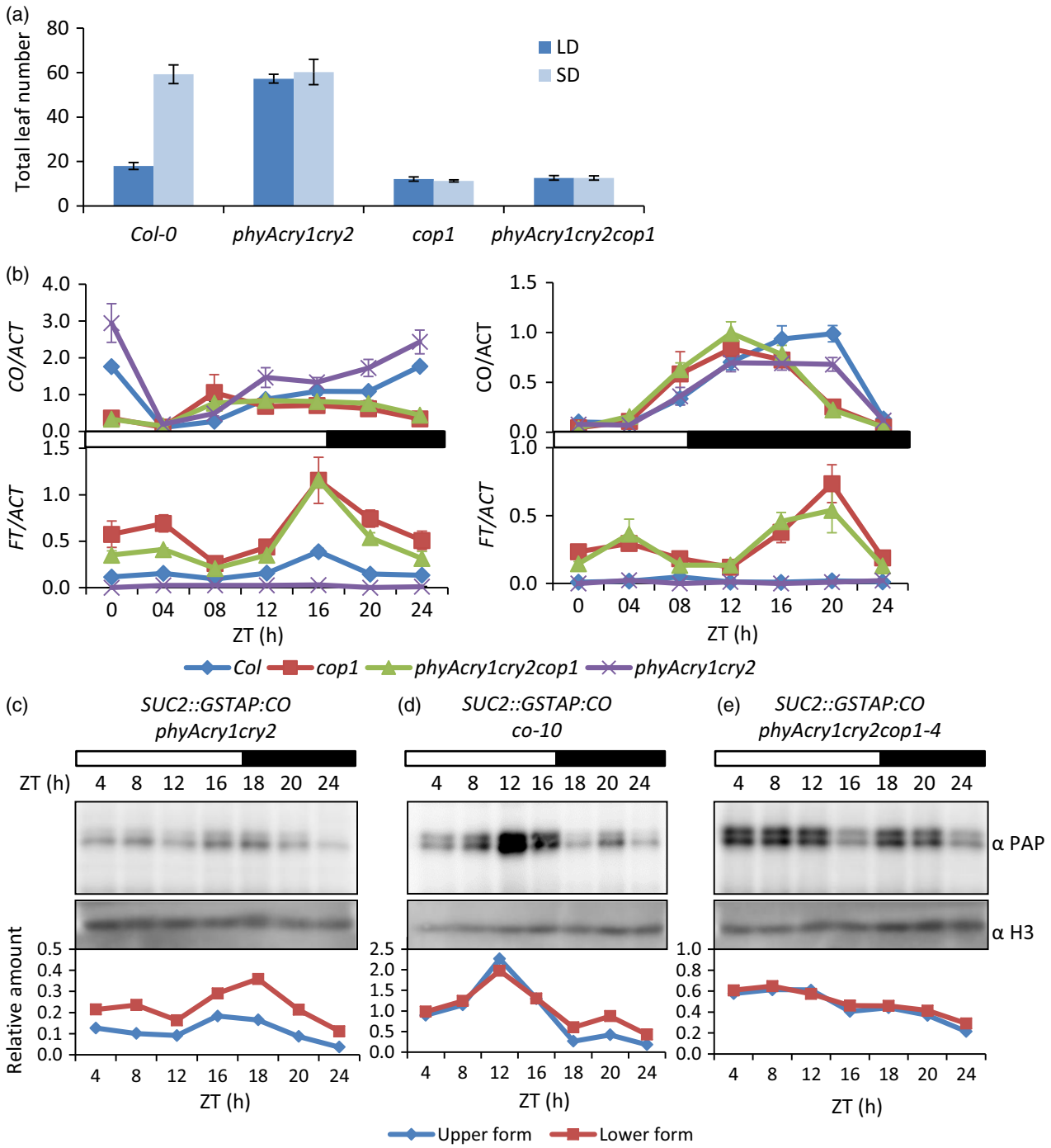

Figure 3. Photoreceptor involvement in post-translational regulation of CONSTANS (CO) protein is through CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1).
(a) *phyA cry1 cry2 cop1* mutant plants flower earlier than Col or *phyA cry1 cry2* and at a similar time to *cop1-4* mutants. Flowering time is expressed as total leaf number at flowering under long day (LD; 16-h light/8-h dark) and short day (SD; 8-h light/16-h dark) conditions. At least 12 plants per genotype were analysed and the mean was calculated ± SD.
(b) Temporal expression profiles of *CO* and *FT* mRNA levels under LDs or SDs. In both *phyA cry1 cry2 cop1* and *cop1* mutants *FT* mRNA levels but not *CO* mRNA levels were increased compared with Col controls. Seedlings were grown for 10 days on soil before harvesting. The vertical axis represents the ratio between *CO* or *FT* mRNA and *Actin* (*ACT*) mRNA ± SD. The horizontal axis is Zeitgeber time (ZT) represented as hours after dawn. White bars illustrate the duration of day, black bars the duration of night.
(c)–(e) Detection on Western blots of GSTAP:CO protein expressed from the *SUC2* promoter in *phyAcry1cry2* mutants (c), *co-10* mutants (d), and *phyAcry1cry2-cop1* mutants (e). Nuclear proteins were extracted and detected using anti-peroxidase–anti-peroxidase complex (PAP) antibody. The relative amount of each form of the protein detected on each Western blot is shown on the vertical axis of each graph. Western blots were quantified using IMAGE-J and signals for each form of CO were divided by its H3 control. The horizontal axis is as in panel b.

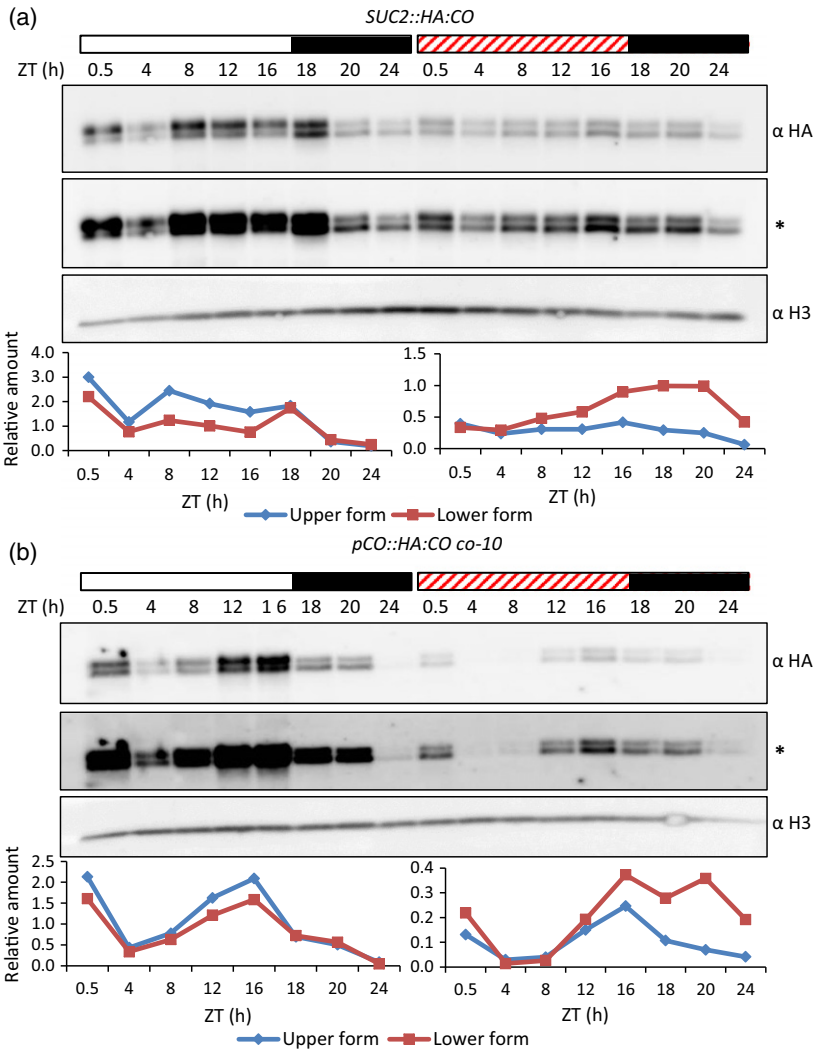

Figure 4. Red light induces degradation of phosphorylated CONSTANS (CO).
(a, b) Diurnal accumulation of CO protein in *SUC2::HA:CO* (a) or *pCO::HA:CO co-10* (b) transgenic plants exposed to white or red light. Seedlings were grown for 10 long days (LDs; 16-h light/8-h dark) under white light and transferred to red or maintained under white light for 16 h followed by 8 h of darkness. Treatments are illustrated by the diagram above each gel. Samples were harvested at the shown times and Western blots of nuclear proteins were performed using anti-HA antibodies to detect HA:CO. Anti-H3 antibodies were used as a control. Asterisks indicate a longer exposure time of the membrane. The relative amount of each form of the protein detected on each Western blot is illustrated in the graphs. Western blots were quantified using ImageJ and signals for each form of CO were divided by its H3 control. The horizontal axis is Zeitgeber time (ZT) represented as hours after dawn. White, striped and black bars illustrate the duration of white light, red light and darkness, respectively.

to day length. In this study, we demonstrated that phosphorylation is a previously unrecognized post-translational modification of CO and that this influences the rate of turnover of the protein (Figure 1). The phosphorylated form was more abundant than the unphosphorylated protein in conditions in which COP1 activity was low, such as in the *cop1* mutant or in wild-type plants grown in W light. By contrast the relative abundance of the two forms was reversed in conditions in which COP1 activity was high, such as in wild-type plants grown in the dark or in triple mutants impaired in the CRY1 CRY2 PHYA photoreceptors. That reduction of the phosphorylated form in the photoreceptor triple mutant was due to increased activity of COP1; this was confirmed by introducing the *cop1* mutation to produce a quadruple mutant and showing that in this background higher levels of phosphorylated CO accumulated. Also in R light, the phosphorylated form was more strongly reduced in abundance than the unphosphorylated form. At least one site of phosphorylation is located in the C-terminal part of the protein that includes the CCT domain. We propose that CO phosphorylation contributes to the photoperiodic response by enhancing ubiquitination of the protein by the COP1–SPA1 E3 ubiquitin ligase complex, thereby ensuring its rapid turnover in the dark.

Phosphorylation of CO is not regulated by light or the circadian clock

In the *cop1* mutant the phosphorylated form of CO is present at equal abundance throughout the day and most of the night. Therefore, the diurnal fluctuation in the abundance of the phosphorylated protein detected in wild-type plants is likely driven by regulated turnover rather than by a diurnal pattern in CO phosphorylation caused by varying activity of a kinase. So the diurnal pattern in abundance of phosphorylated CO therefore has a different basis from that of proteins whose phosphorylation is more directly regulated by light, such as the TFs PHYTOCHROME INTERACTING FACTOR 3 (PIF3) and PIF5 (Al-Sady *et al.*, 2006; Shen *et al.*, 2007).

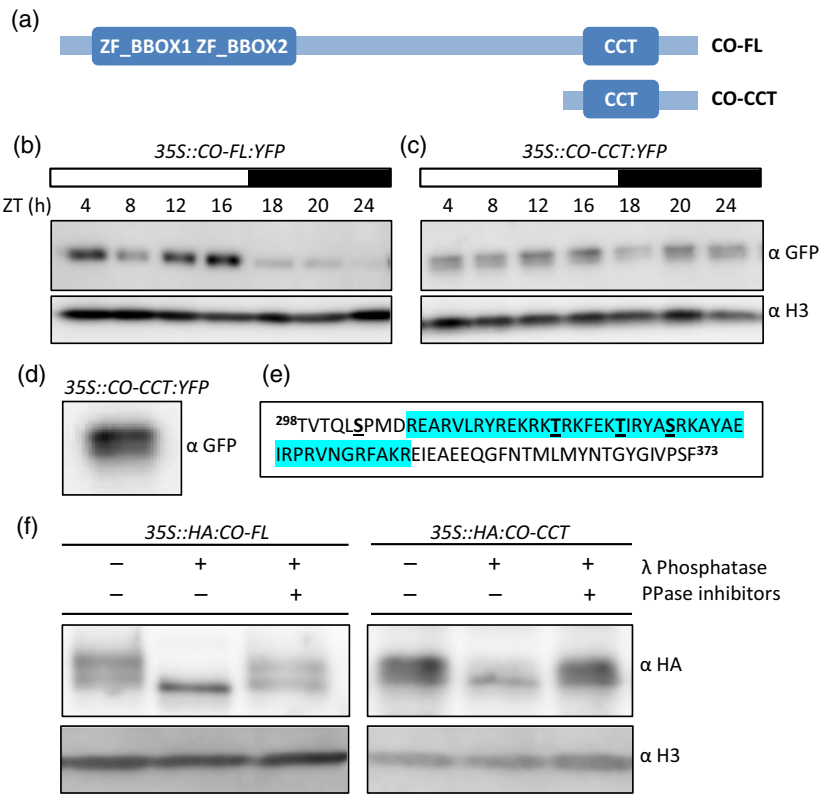

Figure 5. The C-terminal part of CONSTANS (CO) is sufficient for its phosphorylation but not for its dark degradation.
(a) Illustration of the full-length CO protein [CO-FL; 1–373 amino acids (aa)] and the C-terminal truncated protein (CO-CCT; 272–373 aa). The proteins were fused to the N-terminus of yellow fluorescent protein (YFP) and expressed from the *35S* promoter.
(b, c) Western blot analysis of CO-FL:YFP (b) or CO-CCT:YFP (c) nuclear proteins in plants grown under 16-h long days. Light treatments are shown in the diagram above each gel. Anti-GFP antibodies were used to detect CO-FL and CO-CCT and anti-H3 antibodies were used as control.
(d) Western blot analysis of CO-CCT:YFP protein at Zeitgeber time 12.
(e) Amino acid sequence of CO-CCT truncated protein. The CCT domain is highlighted. The NetPhos 2.0 server (http://www.cbs.dtu.dk/services/NetPhos/) was used to identify those residues most likely to be phosphorylated, and these are marked in bold and underlined.
(f) Phosphatase treatment of HA:CO-FL and HA:CO-CCT in infiltrated tobacco leaves. Nuclear proteins were treated with lambda (λ) phosphatase (+) or mock (–) and phosphatase (PPase) inhibitors (+) or mock (–). Anti-HA antibodies were used to detect HA:CO protein and anti-H3 antibodies were used as loading control.

Interestingly, although the relative abundance of the phosphorylated and unphosphorylated forms of CO varied in response to light or in different genetic backgrounds, both forms were detected under almost all conditions. Therefore, either the kinase activity required for CO phosphorylation is limiting so that some non-phosphorylated protein is always present, or its activity is in equilibrium with a phosphatase that maintains a balance of both forms. The phosphorylated form was more dynamic in the rate of its degradation, such as after transfer to the dark, and so maintaining a pool of unphosphorylated protein might be necessary to ensure that CO is not too rapidly ubiquitinated and degraded. For example, in conditions of low light intensity significant COP1 activity may be present during the photoperiod, and if CO was only present in the phosphorylated form it might be rapidly degraded and no protein would be available to activate *FT* transcription.

Phosphorylated CO is the preferred substrate for degradation

In addition to CO, several other COP1 substrates have been described. These include the TFs HY5 (Osterlund *et al.*, 2000), HFR1 (Duek *et al.*, 2004; Jang *et al.*, 2005; Yang *et al.*, 2005), LONG AFTER FAR-RED LIGHT 1 (LAF1) (Seo *et al.*, 2003) and PRODUCTION OF ANTHOCYANIN PIGMENT 1 (PAP1) as well as PAP2 (Maier *et al.*, 2013). Furthermore, the photoreceptors CRY2, PHYA and PHYB are substrates for COP1 (Seo *et al.*, 2004; Jang *et al.*, 2010). Phosphorylation of two of the TF targets was previously shown to influence the efficiency with which they are degraded in a COP1-dependent manner. HY5 is phosphorylated at a serine residue close to the COP1-binding site, and this phosphorylation reduces recognition by COP1. So the unphosphorylated form of HY5 is the preferred COP1 substrate (Hardtke *et al.*, 2000). By contrast, for the basic helix–loop–helix transcription factor HFR1, the preferred COP1 substrate *in vivo* appears to be the phosphorylated form, as the *cop1* mutation increases the relative abundance of phosphorylated HFR1 in the dark (Duek *et al.*, 2004). *In vitro* a truncated HFR1 protein in which the phosphorylated serine residues were mutated was more stable (Park *et al.*, 2008). Therefore, phosphorylation might influence the stability of CO and of other COP1 substrates by altering the strength of the interaction with COP1, as shown for HY5 (Hardtke *et al.*, 2000), or perhaps the rate of ubiquitination by COP1.

The COP1-independent regulation of CO stability

In addition to the COP1–SPA1 ubiquitin ligase complex, other post-translational regulatory steps have been described to regulate the abundance of CO. Interaction with FKF1 stabilizes CO protein in the afternoon of LDs, generating a peak in protein abundance around 12 h after

dawn (Song *et al.*, 2012). Our experiments detect a similar high abundance of CO protein at this time, and the phosphorylated protein is the more abundant form (Figure 2c, d). These experiments suggest that FKF1 stabilizes both forms and may preferentially stabilize the phosphorylated form.

Also, early in the day, CO is degraded through the activities of the R-light photoreceptor PHYB and the HOS1 ubiquitin ligase (Valverde *et al.*, 2004; Lazaro *et al.*, 2012). We found that the phosphorylated form of CO was more labile than the unphosphorylated form under R light as well. The phosphorylated form of CO is therefore the preferred substrate for degradation both via COP1 in the dark and by R-light-induced proteolysis. Thus, the mechanism by which CO protein is post-translationally regulated in the night resembles the one that occurs during the morning in response to R light.

The C-terminal part of CO can be phosphorylated but is not a target for degradation

The C-terminal region, including the CCT domain, was previously suggested to be important for degradation of CO protein, because truncated CO proteins lacking this domain were unable to bind COP1, SPA1 and HOS1 proteins *in vitro* (Laubinger *et al.*, 2006a; Jang *et al.*, 2008; Liu *et al.*, 2008; Jung *et al.*, 2012). In this study, a C-terminal-truncated CO protein (CO-CCT), containing the CCT domain, was found to be present *in vivo* as a phosphoprotein. Phosphorylation often occurs at multiple sites within a single substrate, and this was demonstrated for the COP1 substrate HFR1 (Park *et al.*, 2008), so CO may contain phosphorylated residues at other sites in addition to the C-terminal region. Also, no diurnal regulation of CO-CCT abundance was observed on Western blots. The COP1-binding sites or the lysine residues to which ubiquitin is attached are therefore probably located in other parts of the protein, so that even although it is phosphorylated CO-CCT protein is not efficiently degraded.

CONCLUDING REMARKS

Here we demonstrate that phosphorylation of CO influences its stability. However protein phosphorylation and dephosphorylation can also regulate the activity of proteins (Pawson and Scott, 2005). The two forms of CO might therefore also differ in their activity. Phosphorylation adds a net negative charge to TFs, and this can enhance the rate by which they activate transcription or bind to DNA (Tan and Khachigian, 2009). In this case, the phosphorylated form of CO might be the form that activates transcription of downstream genes, particularly *FT*, as well as the less stable form. The diurnal regulatory pattern of accumulation of CO phosphoprotein, which presents a peak at the same time as the protein activates *FT* transcription, also suggests a role for this phosphorylation in the modulation of CO activity. Impairing phosphorylation of CO will be required to assess its full significance, for example by identifying the phosphorylated residues and introducing mutations. Our demonstration that *in vivo* phosphorylation sites are located close to the C-terminus and previous demonstrations that phosphorylation occurs close to COP1 recognition sites (Hardtke *et al.*, 2000) will help to define phosphorylated residues for such analyses.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

All genotypes used are in Columbia (Col-0) background except the transgenic plants *35S::HA:CO co-2* and *35S::CO phyA cry1 cry2*, which are in the L*er* background. The *co-10* (Laubinger *et al.*, 2006a), *cop1-4* (Deng and Quail, 1992) and *phyA cry1 cry2* (Mockler *et al.*, 2003) mutants were previously described. The *cop1-4* mutant was crossed to *phyA cry1 cry2* to generate the *phyA cry1 cry2 cop1* quadruple mutant. The *35S::CO* transgenic plants were previously described (Valverde *et al.*, 2004). *SUC2::HA:CO* transgenic plants (Jang *et al.*, 2009) were crossed with *cop1-4* mutant to generate *SUC2::HA:CO cop1-4*. Seeds were stratified either in H_2O or on soil for 2–3 days at 4°C in the dark before being transferred to growth chambers under controlled LD conditions (16-h light/8-h dark) or SD conditions (8-h light/16-h dark). Both conditions in Percival growth chambers had a white illumination of 120 μmol m^{-2} sec^{-1} at 20–22°C. For *in vitro* culture, seeds were sterilized in 70% (v/v) ethanol (EtOH) for 1 min followed by 100% (v/v) EtOH for another minute. Thereafter they were stratified in H_2O for 2–3 days at 4°C in the dark before they were sown on Petri dishes with solid germination medium (GM). Plates were transferred to controlled LD or SD conditions of 80 μmol m^{-2} sec^{-1} white light at 22°C. Monochromatic R- or B-light conditions, with illumination of 60 or 40 μmol m^{-2} sec^{-1}, respectively, were produced by LED light sources.

Plasmid constructions

To generate *SUC2::GSTAP:CO*, GFP was replaced by GSTAP (Van Leene *et al.*, 2008) in the *SUC2::GFP:CO* fusion (An *et al.*, 2004) in the binary vector pGreenII 0229 using *Hin*dIII and *Kpn*I restriction enzymes (New England Biolabs, https://www.neb.com/). Verified constructs were introduced into *Agrobacterium tumefaciens* and thereafter into the genotypes *co-10, phyA cry1 cry2* and *phyA cry1 cry2 cop1-4.* Detection of GSTAP:CO on Western blots was performed using anti-peroxidase (PAP) antibody (Sigma, http://www.sigmaaldrich.com/) that recognizes Protein G in the tag. Partial CO (CO-CCT, 298–373 aa) and full-length CO (CO-FL, 1–373 aa) were amplified by PCR, introduced into pDONR201 and inserted into the binary vector pXCNG and pAlligator2 by Gateway reactions (Invitrogen, http://www.invitrogen.com/). Constructs were introduced into *A. tumefaciens* and thereafter into Arabidopsis Col plants.

Flowering-time measurements

Flowering time was measured by counting the total number of leaves, including rosette and cauline leaves, at flowering. At least 12 plants per genotype were used for each measurement, unless stated otherwise, and average (AVG) and standard deviation (SD) were calculated accordingly.

Analysis of gene transcript levels (quantitative RT-PCR)

Twenty seedlings (about 100 μg) per sample were harvested in liquid nitrogen before being homogenized in a Qiagen homogenizer three or four times, each time for 1 min at 30 Hz, to produce a fine powder. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, http://www.qiagen.com/). Samples were treated with DNase (Ambion, http://www.ambion.com/) and 1.5 μg of RNA was used for cDNA synthesis using Oligo-(dT)18 and SuperScript II Reverse Transcriptase enzyme (Invitrogen). The cDNA was diluted 1:10 with H_2O and 3 μl were used as a template for quantitative RT-PCR reactions. Quantification was carried out by the LightCycler® 480 system (Roche, http://www.roche.com/). Error bars represent technical replicates of one biological sample. Primers that were used to amplify *CO* mRNA are 5′-ACGCCATCAGCGAGTTCC-3′ and 5′-AAATGTATGCGTTATGGTTAATGG-3′, for *FT* mRNA 5′-GGTGGAGAAGACCTCAGGAA-3′, for *Actin* (*ACT*) 5′-GGTAACATT GTGCTCAGTGGTGG-3′ and 5′-AACGACCTTAATCTTCATGCTGC-3′, and for *PP2a* 5′-CAGCAACGAATTGTGTTTGG-3′ and 5′-AAA-TACGCCCAACGAACAAA-3′.

Nicotiana benthamiana infiltration

For infiltration of *N. benthamiana*, transgenic *A. tumefaciens* carrying different constructs was cultivated on YEB plates with the appropriate antibiotics. Liquid YEB medium was inoculated with the cell culture and incubated overnight at 28°C. Cells were harvested by centrifugation for 10 min at 2397 ***g*** and mixed with resuspension buffer to a final OD_{600} of 1. Each cell suspension was mixed with the same volume of cell suspension carrying the viral silencing suppressor P19 (Huq *et al.*, 2004; Khanna *et al.*, 2004; Leivar *et al.*, 2008) and acetosyringone (150 μM final concentration). Cell suspensions were incubated for 1 h at 22–25 °C, and subsequently three leaves per plant were infiltrated using a syringe. Plants were incubated for 2–3 days in constant light (LL) before the leaves were harvested and kept in liquid nitrogen until protein was extracted.

Arabidopsis transformation

All plasmids were introduced into *A. tumefaciens* strain GV3101 (pMP90) (Koncz and Schell, 1986) and introduced into plants using the floral dip method (Clough and Bent, 1998).

Transformed plants with GSTAP:CO or CO:YFP constructs were selected with a BASTA solution (glufosinate 200 mg L^{-1}) and plants transformed with the HA:CO constructs were selected for GFP marker in the seed coat. At least three independent homozygous lines were established in the T_3 generation for all constructs that are described in this paper.

Protein extraction and Western blot

The CO protein was detected using antibody against the native protein according to Farrona *et al.* (2011) as well as buffer preparation for extracting all other protein samples. Less plant material was required to detect CO protein using antibodies against an epitope tag: about 20 frozen seedlings were ground to fine powder using the Qiagen homogenizer three or four times, each time for 1 min at 30 Hz, and mixed with 1.2 ml of breaking buffer (Farrona *et al.*, 2011). After 10 min of centrifugation at 2790 ***g*** at 4°C, the supernatant was discarded and 1 ml of 1 × washing buffer (Farrona *et al.*, 2011) was added to the pellet and mixed until it was dissolved. Three more washes with the washing buffer were carried out in decreased centrifugation speeds: 1780, 1000 and 690 ***g*** at 4°C. The pellet was mixed with the same volume (20–50 μl) of 2 × Laemmli buffer (Farrona *et al.*, 2011), heated to 95°C for 10 min, centrifuge for 1 min at 2790 ***g*** and transferred into a new tube. The samples were loaded on 10% polyacrylamide gel and transferred overnight at 4°C to a polyvinylidene difluoride (PVDF) membrane (Sigma). Western blot was carried out using primary antibodies against HA/GFP/Histone H3 and PAP and secondary antibodies against rat/mouse/rabbit HRP or no secondary antibody, respectively. Radioactively labelled proteins after immunoprecipitation (Figure 1c) were separated using 10% Pre-cast NuPAGE Bis-Tris Gel (Invitrogen) with 2-(*N*-morpholine)-ethanesulphonic acid (MES)-SDS buffer. The radioactive gels were transferred to a PVDF, iBlot® Transfer Stack (Invitrogen) using the dry blotting system iBlot. Anti-CO antibodies were used to detect CO proteins.

Phosphatase treatment

Equal amounts of nuclear protein pellets were mixed with λ protein phosphatase (New England Biolabs) and reaction reagents with or without protein phosphatase inhibitor cocktail (ThermoFisher Scientific, https://www.thermofisher.com/). After the samples had been mixed with the reaction materials they were incubated at 30°C for 1 h. Thereafter they were centrifuged for 2 min at 2790 ***g***. Pellets were mixed with 2× Laemmli buffer, heated at 95°C for 10 min, briefly centrifuged and transferred into a new tube. The samples were then loaded on 10% polyacrylamide gel according to the nuclear protein extraction protocol.

Visualization and quantification of the proteins

The membrane was rinsed with a mix of SuperSignal West Dura Chemiluminescent Substrate and SuperSignal West Femto Chemiluminescent Substrate (ThermoFisher Scientific, https://www.thermofisher.com) and images were obtained using a cooled-CCD camera system (Fujifilm LAS-4000). The captured images were quantified with the IMAGE-J 1.43u software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) and normalized to the anti-H3 signal of the same blot.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. CONSTANS protein can be resolved into two forms.

Figure S2. Characterization of different genotypes.

Figure S3. Phosphorylated CONSTANS is preferentially degraded by CONSTITUTIVE PHOTOMORPHOGENIC 1 in the dark.

Figure S4. Mutations in PHYTOCHROME A, CRYPTOCHROME 1 and CRYPTOCHROME 2 reduce the phosphorylated form of CONSTANS.

Figure S5. Diurnal *CO* mRNA levels in the triple *phyA cry1 cry2* and quadruple *phyA cry1 cry2 cop1* mutants.

Figure S6. Phosphorylated CONSTANS is preferentially degraded under red light and stabilized under blue light.

Figure S7. The C-terminal part of CONSTANS is not sufficient for dark degradation.

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