Phosphorylation of CONSTANS promotes its COP1-dependent degradation during photoperiodic regulation of flowering in Arabidopsis

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Significance statement

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Photoperiodic flowering involves light-regulated post translational modification of CONSTANS transcription factor, conferring degradation of the protein in darkness and allowing its accumulation in light. Here we add to current models by demonstrating that CONSTANS is also phosphorylated, and that this increases the rate of its turnover in darkness dependent on the activity of the CONSTITUTIVE PHOTOMORPHOGENIC 1 ubiquitin ligase complex.

Summary (250 words)

Seasonal flowering involves responses to changing day length. In *Arabidopsis thaliana*, the CONSTANS (CO) transcription factor promotes flowering in long days of spring and summer. Late flowering in short days is due to instability of CO, which is efficiently ubiquitinated in darkness by 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 darkness. These changes in relative abundance require COP1, because in the *cop1* mutant the phosphorylated form is always more abundant. Inactivation of the Phytochrome A Cryptochrome 1 Cryptochrome 2 photoreceptors in the *phyA cry1 cry2* triple mutant reduces most strongly the abundance of the phosphorylated form so that unphosphorylated CO is the 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.

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 changing 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 (LD) of 16 h light than to short days (SD) of 10 h or 8 h (Redei 1962). Mutants that impair photoperiodic responses were screened for by identifying plants that flowered later than wild-type under LD, but that were unaffected in their flowering time under SD (Koornneef *et al.* 1991, Redei 1962). These mutants defined a genetic pathway termed the long-day or photoperiodic flowering pathway. The CONSTANS (CO) transcription factor plays a central role in this pathway and its abundance is higher under LD 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 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.

CO is a member of the B-box zinc finger family (Khanna *et al.* 2009). It contains two B-boxes near the C-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, the protein contains at the C-terminus a CONSTANS, CONSTANS-LIKE, TIMING OF CAB1 domain that mediates DNA

binding and is functionally important based on analysis of mutant alleles (Robson*, et al.* 2001, Strayer *et al.* 2000). CO directly binds to motifs in the proximal promoter of its major target gene *FLOWERING LOCUS T* (*FT*) (Song *et al.* 2012, Tiwari *et al.* 2010). These motifs are related to those recognized by other CCT domain proteins and are required for *FT* transcription in LDs (Adrian *et al.* 2010, Gendron *et al.* 2012). Transcription of *CO* occurs in the vascular tissue and its misexpression from phloem specific promoters, such as that of the *SUCROSE TRANSPORTER 2* (*SUC2*) gene, is sufficient to promote flowering and complement the *co* mutation (An *et al.* 2004, Takada and Goto 2003). *FLOWERING LOCUS T* (*FT*) is also expressed in the vascular tissue in a CO-dependent manner (Adrian*, et al.* 2010, Takada and Goto 2003). 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 *FT* transcription in the vascular tissue, suggesting that CO activity is restricted at the post-transcriptional level to these cells (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 to the formation of 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 promotes *FT* transcription only under LDs. At one level this occurs through *CO* transcriptional regulation. 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 in LDs, whereas under SDs a high amplitude peak in *CO* mRNA occurs exclusively in darkness. 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 transcription factors that are direct repressors of *CO* transcription (Fornara *et al.* 2009, Imaizumi*, et al.* 2005, Sawa *et al.* 2007). 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 darkness, CO is degraded by the 26S proteasome following ubiquitination by the CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) (Jang *et al.* 2008, Liu *et al.* 2008) and SUPPRESSOR OF PHYA1 (SPA1) (Jang*, et al.* 2008, Laubinger *et al.* 2006, 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 CRYPTOCHROME 1 (CRY1) and CRYPTOCHROME 2 (CRY2) photoreceptors to SPA1 in a blue (B) light dependent manner (Lian *et al.* 2011, Liu *et al.* 2011, Zuo *et al.* 2011). PHYTOCHROME A (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, a direct interaction between CO and the ubiquitin ligase FKF1 increases CO levels (Song*, et al.* 2012). Direct interaction between these proteins stabilizes, by an unknown mechanism, CO in the light under LDs (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 (Jang*, et al.* 2008, Valverde*, et al.* 2004). This reduction of CO levels by PHYB was suggested to 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 transcription factor stability and activity (Hill and Treisman 1995, Hunter and Karin 1992, Sugiyama *et al.* 2008). In plants, transcription factors that contribute to photoreceptor signaling pathways, the oscillator of the circadian clock and clock-controlled output pathways are regulated by phosphorylation (Bu *et al.* 2011, Duek *et al.* 2004, Fujiwara *et al.* 2008, Hardtke *et al.* 2000, Sugano *et al.* 1999). Phosphorylation of a protein can influence the efficiency with which it is ubiquitinated and the rate of ubiquitination, and degradation of several COP1 transcription factor substrates are altered by phosphorylation (Hoecker 2005). For example, the transcription factors (TFs) LONG HYPOCOTYL 5 (HY5) and LONG HYPOCOTYL IN FAR RED 1 (HFR1) are phosphorylated, and this modification influences their rate of degradation mediated by COP1 (Duek*, et al.* 2004, Hardtke*, et al.* 2000, Park *et al.* 2008). The effect of phosphorylation on the turnover of these COP1 substrates differs. While the unphosphorylated HY5 protein is more efficiently ubiquitinated and degraded in the dark and is functionally more active than the phosphorylated form, the phosphorylated form of HFR1 is more active and less abundant in the dark.

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 darkness in a process

dependent on COP1 ubiquitin ligase and also under R light. We propose that post-translational regulation by phosphorylation contributes to instability of CO in darkness and under SD and thereby to 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 made from *35S::CO* transgenic plants (Figure 1A). The *35S* promoter is active in most cell types whereas the *CO* promoter is mainly active in the 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 HA:CO is expressed specifically in the phloem companion cells where it promotes flowering, and *pCO::HA:CO* plants, where it is expressed under its native promoter. The *pCO:HA:CO* transgene complements the late flowering-time phenotype of *co-10* mutants and *CO* and *FT* mRNAs exhibit diurnal expression in these plants confirming that the promoter and protein fusions retain activity (Figure S1). 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). 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 extracted 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 represents the slower migrating one.

Further evidence for CO phosphorylation was obtained from *in vivo* labelling experiments utilizing gamma labelled ATP. Transgenic *35S:HA:CO co-2* plants and wild-type Landsberg *erecta* controls were transferred to medium containing gamma labelled ATP for three days. Protein was extracted and immunoprecipitated with HA antibody. The immunoprecipitated protein was then separated by electrophoresis and part of the gel was exposed to a film whereas the other was transferred to a membrane and probed with HA 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 WT and *cop1-4* mutant plants growing under LDs of 16h light 8h dark to determine whether one of the forms of CO is preferentially degraded in the dark. After 16h in 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 hours in darkness, 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 the more abundant, whereas in *SUC2::HA:CO cop1-4* the phosphorylated form was the more abundant, as observed during light exposure (Figure 2A; Figure S2). Quantification of these western blots, indicated that the ratio of phosphorylated to unphosphorylated CO forms was higher than one in both genotypes after 16h of light, whereas in *SUC2::HA:CO* the ratio changed to favor the unphosphorylated form after 8h in dark and in *SUC2::HA:CO cop1-4* the ratio remained approximately the same 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 CO diurnal regulation patterns in both *pCO::HA:CO co-10* and *SUC2::HA:CO* plants grown under 16 h LD 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 darkness (Figures 2C, D). For example, comparison of the 12h and 18h time points illustrates the relative reduction in abundance of the phosphorylated form on transfer to darkness. Remarkably, in *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 involvement in post-translational regulation of CO protein is through COP1

Photoreceptors increase CO protein levels 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 analyzed (Figure 3B). The abundance of *FT* mRNA, but not of *CO* mRNA, was strongly reduced in the *phyA cry1 cry2* triple mutant under both LD and SD (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 therefore perhaps CO protein activity. To further test for a correlation 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. Diurnal abundance of the two forms of CO in the different transgenic backgrounds was examined (Figures 3C, D, E). In the absence of the three photoreceptors, GSTAP:CO protein levels were reduced in the afternoon of LDs compared to *SUC2::GSTAP:CO co-10* (Figures 3C, D). Similar results were obtained with 35S:CO (Figure S3). 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 (Kusakina and Dodd 2012, Shen *et al.* 2009), or that the hyper activity of COP1 in those plants contributes to the relative reduction of phosphorylated CO protein levels. 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 low levels and unphosphorylated CO should accumulate to high levels. However, in *SUC2::GSTAP:CO phyA cry1 cry2 cop1* plants both forms of CO strongly

accumulated at all of the time points that the protein was detected (Figure 3E). Thus the photoreceptors probably do not directly promote CO phosphorylation, 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 to the triple photoreceptor mutants was due to post-translational regulation of the protein, *CO* mRNA levels were quantified in the different transgenic plants (figure S4). In contrast to protein levels, *CO* mRNA levels were lower in the *SUC2::GSTAP:CO phyA cry1 cry2 cop1* plants compared to the triple mutant. Taken together, these experiments support the idea that the photoreceptors increase CO protein abundance at the post-transcriptional level by repressing COP1 activity and that this also increases the ratio of phosphorylated to unphosphorylated CO protein.

Red-light mediated degradation reduces the phosphorylated form of CO

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 16h LD of R or white (W) light in plants that express *HA:CO* from *SUC2* or the *CO* native promoter (Figure 4A-B). The overall levels of HA:CO protein were reduced compared to plants exposed to white light (Figures 4A-B). Particularly, the abundance of the phosphorylated form was lower, so that the unphosphorylated form generally appeared the 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 S5). The ratio of phosphorylated to unphosphorylated form was greater than 1.0 for plants grown

under white light, but lower than 1.0 for those grown under R light (Figure S5). Thus, these results indicate that also under R light, the preferred substrate for degradation is the phosphorylated form of CO.

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

CO protein is composed of three major domains, N-terminal zinc-finger B-box domain, a middle region and the C-terminal CCT domain (Putterill*, et al.* 1995, Robson*, et al.* 2001, Strayer*, et al.* 2000). 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.* 2006), because a truncated protein lacking this domain (1-331aa) was unable to bind these proteins *in vitro*. Nevertheless, a short C-terminal version of CO (272-373aa) was not sufficient to interact with SPA1 (Laubinger*, et al.* 2006). To better understand the importance of this domain to the post-translational regulation of CO *in vivo*, a C-terminal region (297-373aa) that includes the CCT domain (CO-CCT) was fused to the N-terminus of YFP and expressed in WT 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 to that of full-length CO (CO-FL:YFP) during a 16 h LD diurnal cycle (Figures 5B-C, S6). In contrast to CO-FL:YFP, which demonstrated a sharp reduction in abundance in darkness (Figure 5B), CO-CCT:YFP was present at similar levels in light and in 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 darkness. However, western blot analysis of CO-CCT:YFP revealed two forms of the protein, suggesting that it was phosphorylated (Figure 5C, D). 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 5E). Two forms of both proteins were detected in this transiently expressed protein. The upper form of both HA:CO-FL and HA:CO-CCT extracted from *N. benthamiana* nuclei was 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 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 a *cop1* mutant or in wild-type plants grown in white light. By contrast the relative abundance of the two forms was reversed in conditions in which COP1 activity was high, such as wild-type plants grown in darkness 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 COP1 activity 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 and 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. The diurnal pattern in

abundance of phosphorylated CO therefore has a different basis than for proteins whose phosphorylation is more directly regulated by light, such as the transcription factor PHYTOCHROME INTERACTING FACTOR 3 (PIF3) and PIF5 (Al-Sady *et al.* 2006, Shen *et al.* 2007).

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 darkness, 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 is present during the photoperiod, and if CO was only present in the phosphorylated form it might be rapidly degraded so that no protein is available to activate transcription of *FT*.

Phosphorylated CO is the preferred substrate for degradation

In addition to CO, several other COP1 substrates have been described. These include the transcription factors LONG HYPOCOTYL 5 (HY5) (Osterlund *et al.* 2000), LONG HYPOCOTYL IN FAR RED LIGHT 1 (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 (Jang *et al.* 2010, Seo *et al.* 2004). Phosphorylation of two of the transcription factor 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 bHLH 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.

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 CO abundance. Interaction with FKF1 stabilizes CO protein in the afternoon, 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). We found that the phosphorylated form of CO was more labile than the unphosphorylated form also under R light. 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 confers phosphorylation but reduced degradation

The C-terminal region including the CCT domain was previously suggested to be important for CO protein degradation, because truncated CO proteins lacking this domain were unable to bind to COP1, SPA1 and HOS1 proteins *in vitro* (Jang*, et al.* 2008, Liu*, et al.* 2008). In this study, a C-terminal truncated protein of CO (CO-CCT) containing the CCT domain was found to be present *in vivo* as a phospho-protein. 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 contributes negative charge to transcription factors, and this can enhance the rate with which they activate transcription or bind to DNA (Tan and Khachigian 2009). In this case, the phosphorylated form of CO might both be the form that activates transcription of downstream genes, particularly *FT*, and the less stable form. The diurnal regulation

pattern of CO phosphoprotein with a peak at the same time as the protein activates *FT* transcription also suggests a role of 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 carboxy-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 plants used in this research are in *Columbia* (Col-0) ecotype background except the transgenic plants *35S::HA:CO co-2* and *35S::CO phyAcry1cry2*, which are in *Landsberg erecta* (L.er) background. *co-10* (Laubinger 2006), *cop1-4* (Deng and Quail 1992) and *phyAcry1cry2* (Pablo D. Cerdán, The University of Buenos Aires, Argentina) mutants were previously described. *cop1-4* mutant was crossed into *phyAcry1cry2* to generate *phyAcry1cry2cop1* quadruple mutant. *35S::CO* transgenic plants were previously described (Valverde 2004). *SUC2::HA:CO* transgenic plants (Jang 2009) were crossed with *cop1-4* mutant to generate *SUC2::HA:CO cop1-4*. Plants grown on soil were stratified either in H_2O or on soil for 2-3 days at 4^oC in darkness before transferred to growth chambers with controlled long-day (LD) conditions, 16 h light/8 h dark, or short-day (SD) conditions, 8 h light/16 h dark. Both conditions in percival growth chambers had a white illumination of 120 $\mu mol/m^{-2}sec^{-1}$ at 20-22^oC. Seeds were sterilized in 70% EtOH for one minute followed by 100% EtOH for another one minute. Thereafter they were stratified in H_2O for 2-3 days at 4^oC in darkness before they were sown on petri dishes with solid germination medium (GM). Plates were transferred to controlled LDs or SDs conditions of 80 µmol/m-2sec-1 white light and 22°C. Monochromatic R, FR and B light conditions were produced by LED light sources.

Plasmid constructions

To generate *SUC2::GSTAP:CO*, GFP was replaced by GSTAP in the *SUC2::GFP:CO* construct (An et al. 2004) in the binary vector pGreenII 0229 using HindIII and KpnI restriction enzymes (New England Biolabs). Verified constructs were

introduced into Agrobacterium tumefaciens and thereafter into the genotypes: *co-10, cop1-4, phyAcry1cry2, phyAcry1cry2cop1-4.* Partial CO construct (CO-CCT, 298-373aa) and CO full-length protein (CO-FL, 1-373aa) were amplified by PCR reaction, introduced into pDONR201 and thereafter inserted into the binary vector pXCNG and pAlligator2 by GW reaction (Invitrogen). Verified constructs were introduced into Agrobacterium 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 list 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)

20 seedlings (about 100µg) per sample were harvested in liquid nitrogen before homogenized 3-4 times into fine powder by Qiagen homogenizer (1 minute at 30 1/s each time). Total RNA was extracted according to manual of RNeasy Plant Mini Kit (QIAGEN). Samples were treated with DNAse (Ambion) and 1,5µg 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 3µl were used as a template for qRT-PCR reaction that was carried out by the LightCycler® 480 system (Roche). Error bars represent technical replicates of one biological repeated experiment, with similar results. Ingredients and volumes per reaction and primers sequences are listed below.

***Nicotiana benthamiana* infiltration**

For infiltration of *Nicotiana benthamiana*, transgenic *Agrobacterium tumefaciens* carrying different constructs were cultivated on YEB plates with the appropriate antibiotics. Liquid YEB medium was inoculated with a touch of cell culture and incubated overnight at 28°C. Cells were harvested by centrifugation for 10 minutes at 4000 rpm and mixed with resuspension buffer to a final OD600nm of 1. Each cell suspension was mixed with the same volume of a cell-suspension carrying the viral silencing suppressor P19 (Huq *et al.* 2004, Khanna *et al.* 2004, Leivar *et al.* 2008) and acetociringone (150µM final concentration). Cell suspensions were incubated for 1 h at room temperature 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 till protein extraction took place.

Arabidopsis transformation

All plasmids were introduced into Agrobacterium strain GV3101 (pMP90) (Koncz and Schell, 1986) and transformed into different plants by floral dip (Clough and Bent, 1998). The first generation of seeds from the dipped plants were sown on soil and after the first two true leaves appeared seedlings were sprayed with a BASTA solution of Glufosinat 200 mg/l (BASTA) to select those plants that contained the BASTA resistance gene. T2 seeds were sown on GM medium and PPT to analyze their segregation. Lines that showed a segregation ratio between 1:2 and 1:4 (based on 40-60 seedlings) were considered for further analysis. At least 3 independent homozygous lines were established in the T3 generation for all constructs that are described in this thesis. Alternatively, for constructs that contained GFP marker in the seed coat, same segregation analysis was performed but seeds were first selected under fluorescent microscope before they were sown on soil.

Protein extraction and western blot

Detection of CO protein using its native antibody was done according to Farrona et al., 2011 as well as buffer preparation for extracting all other protein samples. To detect CO protein by tag-epitope antibodies, about 20 frozen seedlings were grounded to fine powder by Qiagen homogenizer (1 minute at 30 1/s each time) 4-5 times and mixed with 1.2 ml of breaking buffer (Farrona et al., 2011). After 10 minutes of centrifugation at 5000 rpm at 4^{o}C, the supernatant was discarded and 1ml of 1X washing buffer (Farrona et al., 2011) was added to the pellet and mixed together till it was dissolved. 3 more washes with the washing buffer were carried out in decreased centrifugation speeds: 4000, 3000 and 2500 rpm at 4^{o}C. The pellet was mixed with the same volume (20-50µl) of 2X Laemmli buffer (same protocol as above), heated to 95^{o}C for 10 min, shortly centrifuged and transferred into new tube. The samples were loaded on 10% polyacrylamid gel and transferred over-night at 4^{o}C to PVDF membrane. Western blot was carried out using primary antibodies against HA/GFP/H3 and PAP and secondary antibodies against rat/mouse/rabbit HRP or no secondary antibody respectively.

Phosphatase treatment

Equal amounts of pellets of nuclear proteins were mixed with λ protein phosphatase (New England Biolabs) and reaction reagents or λ phosphatase, protein phosphatase inhibitor cocktail (ThermoFisher Scientific) and reaction reagents or just reaction reagents. After the samples were mixed with the reaction materials they were incubated at 30^{o}C for 1 hour. Thereafter they were centrifuged for 2 minutes at 2500 rpm. Obtained pellets were mixed with 2X Laemmli buffer, heated to 95^{o}C for 10 min, shortly centrifuged and transferred into new tube. The samples were then loaded on 10% polyacrylamid 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 FemtoChemiluminescent Substrate (Pierce) and image was obtained using a cooled-CCD camera system (Fujifilm LAS-4000). The captured image was quantified with the Image-J 1.43u software (Wayne Rasband National Institutes of Health, USA) and normalized to the anti-H3 signal of the same blot.

Figure Legends.

Figure 1: CO protein is phosphorylated

(A) Two forms of nuclear CO were detected in transgenic Arabidopsis seedlings expressing *CO* under the constitutive *CaMV 35S* promoter (*35S*), the phloem companion cell specific promoter (*SUC2*) and *CO* native promoter (*pCO*). CO and HA:CO proteins were detected by anti CO and anti HA antibodies respectively. Neither form was detected in *co-10* or Columbia mutant 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 loading control.

(C) *In vivo* labeling of CO protein with radioactive phosphate. Nuclear proteins were extracted from Arabidopsis seedlings, treated for three days with gamma labeled 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). Asterisk marks non-specific bands that appeared in all of the samples, including the controls.

Figure 2. Phosphorylated CO is preferentially degraded by COP1 in the dark.

(A) HA:CO nuclear protein levels detected by western blot at dusk (ZT 16) or dawn (ZT 24) of LDs. Transgenic *SUC2::HA:CO* seedlings, grown under 16 h LDs, accumulate high levels of phosphorylated CO at dusk but not at dawn whereas in the background of *cop1-4* mutant phosphorylated CO levels remain high.

(B) Ratio between 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 3 independent experiments were quantified with Image-J program. Signals for bands representing each form of 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 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 with Image-J program and signals for each form of CO were divided by the signal for the phosphorylated form at ZT 12 of the same genotype.

Figure 3: Photoreceptor involvement in post-translational regulation of CO protein is through COP1.

(A) *phyAcry1cry2cop1* mutant plants flower earlier than Col or *phyAcry1cry2*, similar to *cop1-4* mutants. Flowering time is expressed as total leaf number at flowering under LD and SD conditions. At least 12 plants per genotype were analyzed and the mean was calculated ± SD.

(B) Temporal expression profiles of *CO* and *FT* mRNA levels under LDs or SDs. Both in *phyAcry1cry2cop1* and in *cop1* mutants *FT* mRNA levels but not *CO* mRNA levels were increased compared to Columbia controls. Seedlings were grown for 10 days on soil before harvesting. Average mean represents the ratio between *CO* or *FT* and *Actin* (*ACT*) ± SD. White bars illustrate duration of day, black bars duration of night.

(C-E) Detection of GSTAP:CO protein, expressed from *SUC2* promoter, in *phyAcry1cry2* mutant plants (C) *co-10* mutant plants *(D) and phyAcry1cry2cop1* mutant plants (E) on western blot. Nuclear proteins were extracted and detected by Anti-Peroxidase anti-Peroxidase complex (PAP) antibody.

Figure 4: Red light induces degradation of phosphorylated CO.

(A-B) CO protein diurnal accumulation in *SUC2::HA:CO* (A) or *CO::HA:CO co-10 (B)* transgenic plants. Seedlings were grown for 10 long days under white light and transferred to red or maintained under white light for 16 hours followed by 8 hours of darkness. Samples were harvested at the mentioned times and western blots of nuclear proteins were performed using antibodies against HA to detect HA:CO. Anti-H3 antibodies were used as control. Asterix marks longer exposure time of the membrane.

Figure 5. The C-terminal part of CO is sufficient for its phosphorylation but not for its dark degradation.

(A) Illustration of CO full length protein (CO-FL, 1-373aa) and the C-terminal truncated protein (CO-CCT, 272-373aa). The proteins were fused to the N-terminus of YFP.

(B-C) Western blot analysis of CO-FL:YFP (B) or CO-CCT:YFP (C) nuclear proteins in a 24 h light and dark cycle. Anti-GFP antibody was 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 ZT-12.

(E) Phosphatase treatment of HA:CO-FL and HA:CO-CCT in infiltrated Nb leaves. Nuclear proteins were treated with Lambda (λ) phosphatase (+) or mock (-) and phosphatase (PPase) inhibitors (+) or mock (-). Anti-HA antibodies were used to detect CO protein and anti-H3 antibodies were used as loading control.

Supplementary data

Figure S1. Genetic characterization of different genotypes.

(A-B) Temporal expression of *CO* (A) and *FT* (B) mRNA levels quantified by RT-PCR at indicated ZTs. Seedlings were grown for 10 days on soil before harvesting. Average mean represents the ratio between *CO* or *FT* and *PP2A* ± SD. White bars illustrate duration of day, black bars duration of night.

(C) Flowering-time phenotype of transgenic or Col plants grown for 3 weeks under LDs before pictures were taken.

(D) Flowering time of the same genotypes shown in (C) is expressed as total leaf number at flowering under LD and SD conditions. At least 12 plants per genotype were analyzed and the mean was calculated ± SD.

Figure S2: Phosphorylated CO is preferentially degraded by COP1 in the dark.

HA:CO protein accumulation in *SUC2::HA:CO* or *SUC2::HA:CO cop1-4* transgenic plants at dusk (ZT 16) or dawn (ZT 24). Same western blot as in figure 2A but with a longer exposure time.

Figure S3. Mutations in PhyA, Cry1 and Cry2 reduce the phosphorylated form of CO.

Diurnal rhythm of CO protein accumulation in *35S::CO phyAcry1cry2* plants compared to *35S::CO* under LD conditions.

Figure S4: Photoreceptor involvement in post-translational regulation of CO protein is through COP1.

Temporal expression of *CO* mRNA levels quantified by RT-PCR at indicated ZTs. Samples were grown under LD conditions for 10 days before they were harvested and mRNA was extracted. Average mean represents the ratio between *CO* and *PP2A* ± SD.

Figure S5: Red light initiates the degradation of phosphorylated CO.

(A-B) HA:CO protein accumulation in *SUC2::HA:CO or pCO::HA:CO co-10* transgenic plants under white (W) or red (R) light conditions. Seedlings were grown for 10 days under W light and transferred to R or remained under W light

for 12 hours. Western blots (B) of nuclear proteins were performed and antibodies against HA were used to detect HA:CO. Anti-H3 antibodies were used as control. Three different exposures of the same western blot are shown for HA:CO protein.

Asterisks represent longer exposure times. Western blots of 3 independent experiments were quantified with Image-J program and each form of CO was divided by its H3 control before the ratio was calculated. Numbers represent the mean and error bars show ± SD.

Figure S6. The C-terminal part of CO is not sufficient for dark degradation.

Western blot analysis of 35S::CO-CCT:YFP line #7.7.4 in a 24h light and dark cycle.

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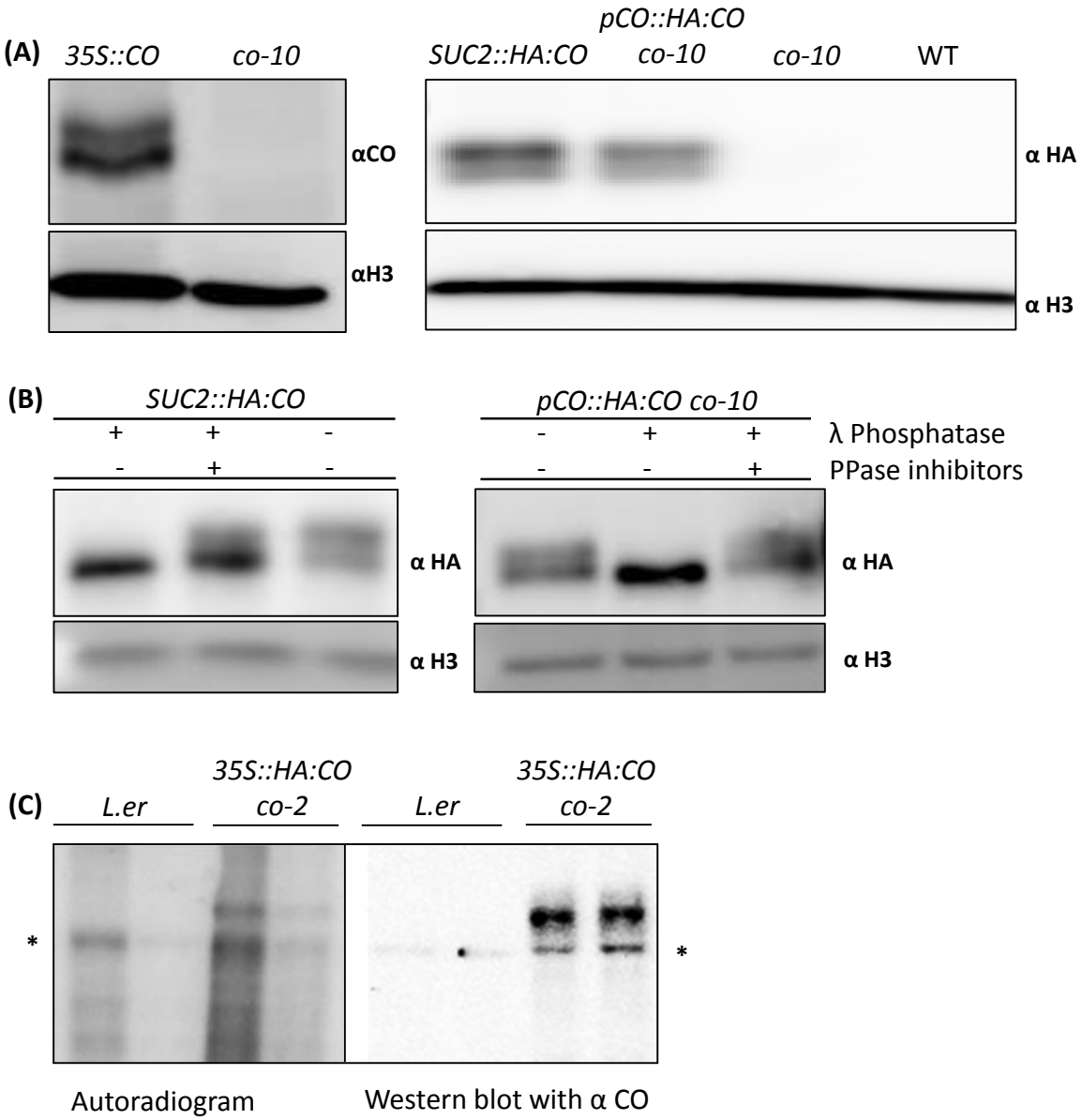
Figure 1


(A)
35S::CO
co-10
αCO
αH3
pCO::HA:CO
SUC2::HA:CO
co-10
co-10
WT
α HA
α H3
(B)
SUC2::HA:CO
pCO::HA:CO co-10
+ + -
- + -
- + +
- - +
λ Phosphatase
PPase inhibitors
α HA
α H3
α HA
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(C)
L.er
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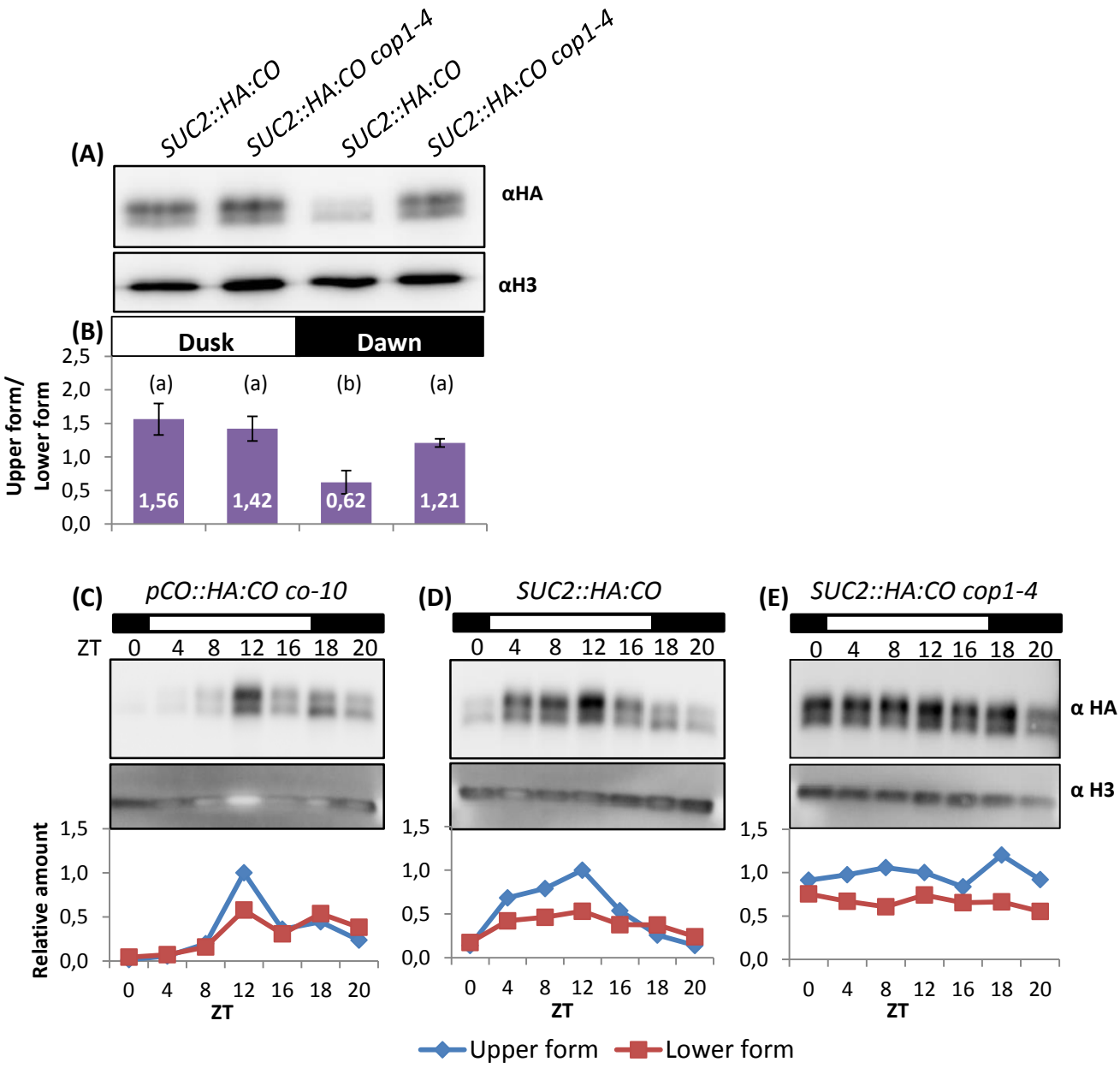
Autoradiogram
Western blot with α CO

Figure 2


SUC2::HA:CO
SUC2::HA:CO cop1-4
SUC2::HA:CO
SUC2::HA:CO cop1-4
(A)
αHA
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(B)
Dusk
Dawn
Upper form/ Lower form
2,5
2,0
1,5
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(a)
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1,56
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1,21
(C)
pCO::HA:CO co-10
(D)
SUC2::HA:CO
(E)
SUC2::HA:CO cop1-4
ZT 0 4 8 12 16 18 20
α HA
α H3
Relative amount
1,5
1,0
0,5
0,0
ZT
Upper form
Lower form

Figure 3

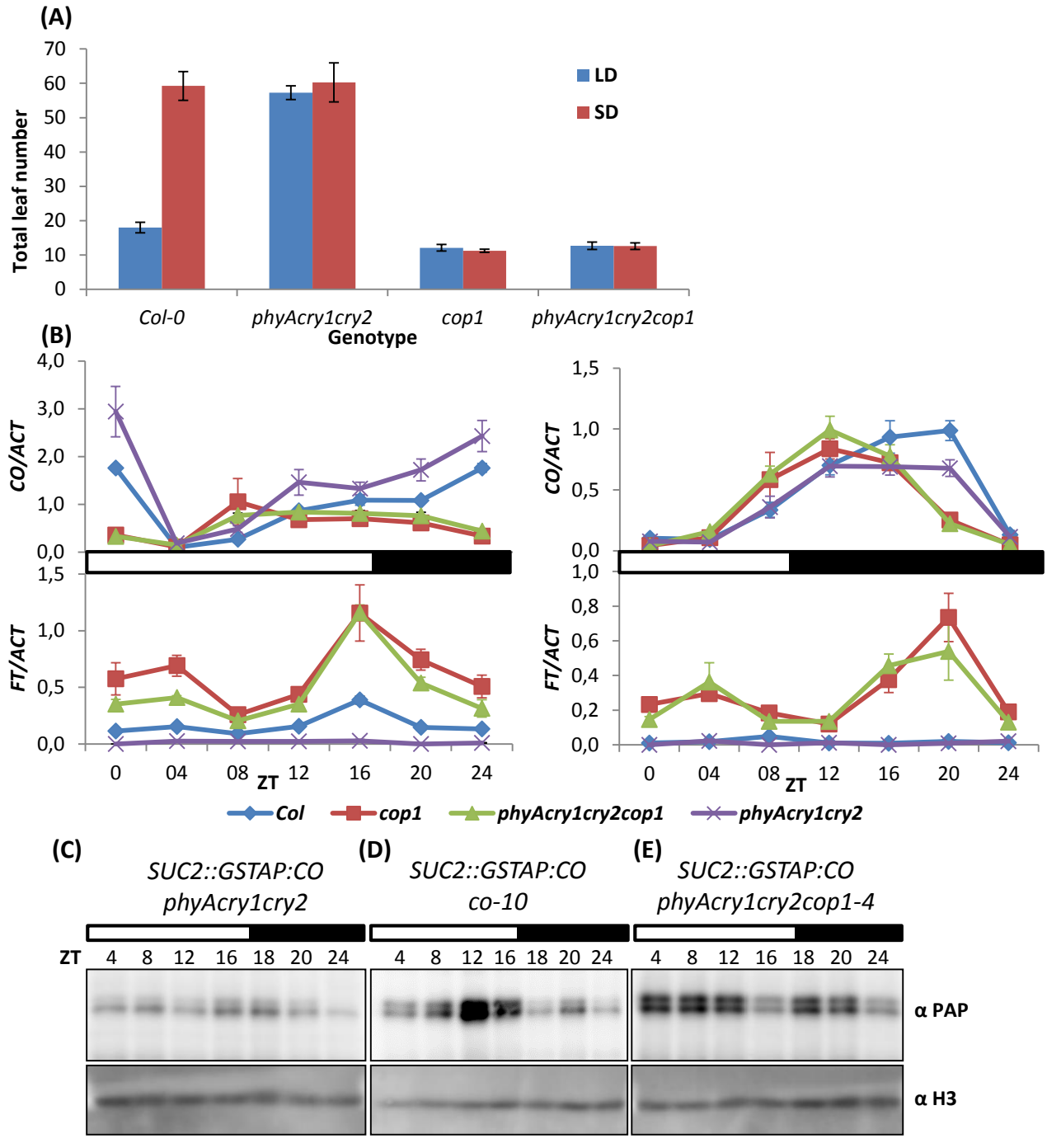
(A)
Total leaf number
LD
SD
Col-0
phyAcry1cry2
cop1
phyAcry1cry2cop1
Genotype
(B)
CO/ACT
FT/ACT
ZT
Col
cop1
phyAcry1cry2cop1
phyAcry1cry2
(C)
SUC2::GSTAP:CO
phyAcry1cry2
(D)
SUC2::GSTAP:CO
co-10
(E)
SUC2::GSTAP:CO
phyAcry1cry2cop1-4
ZT 4 8 12 16 18 20 24
α PAP
α H3

Figure 4

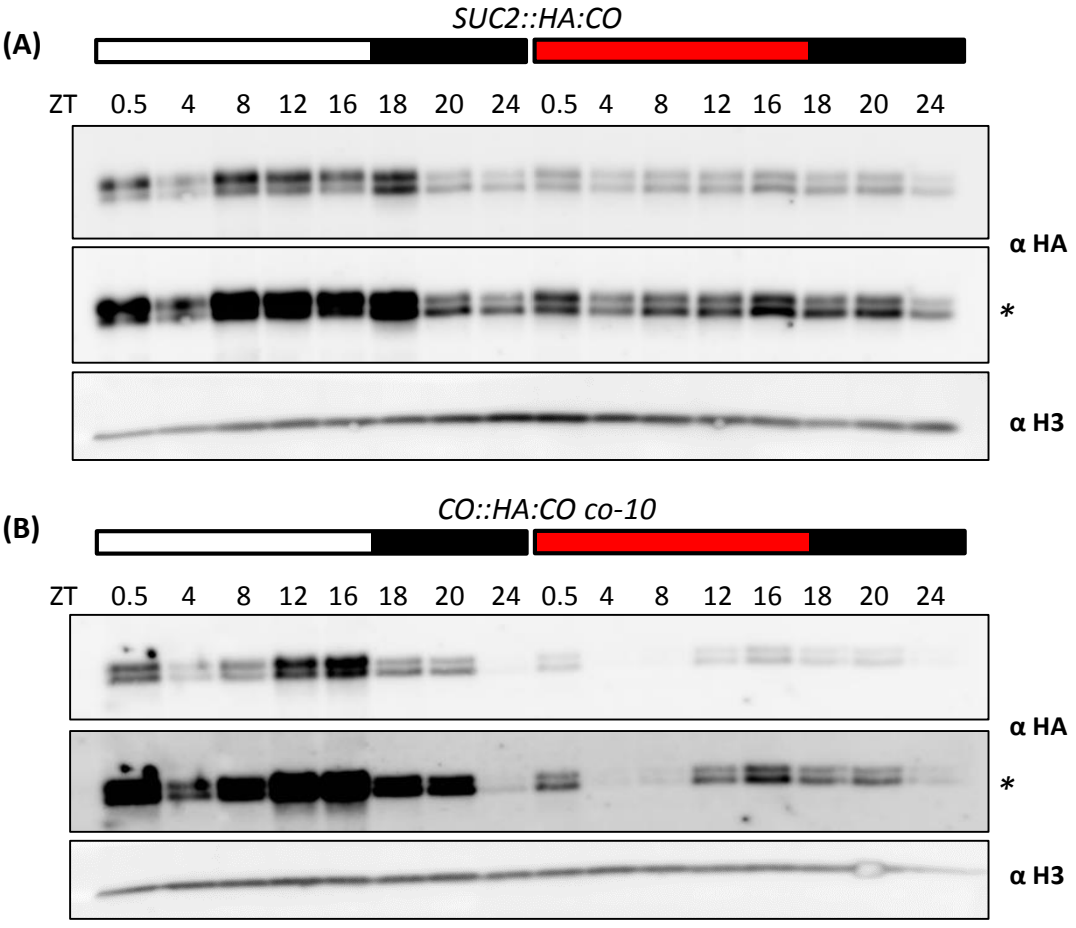
(A)
SUC2::HA:CO
ZT 0.5 4 8 12 16 18 20 24 0.5 4 8 12 16 18 20 24
α HA
*
α H3
(B)
CO::HA:CO co-10
ZT 0.5 4 8 12 16 18 20 24 0.5 4 8 12 16 18 20 24
α HA
*
α H3

Figure 5

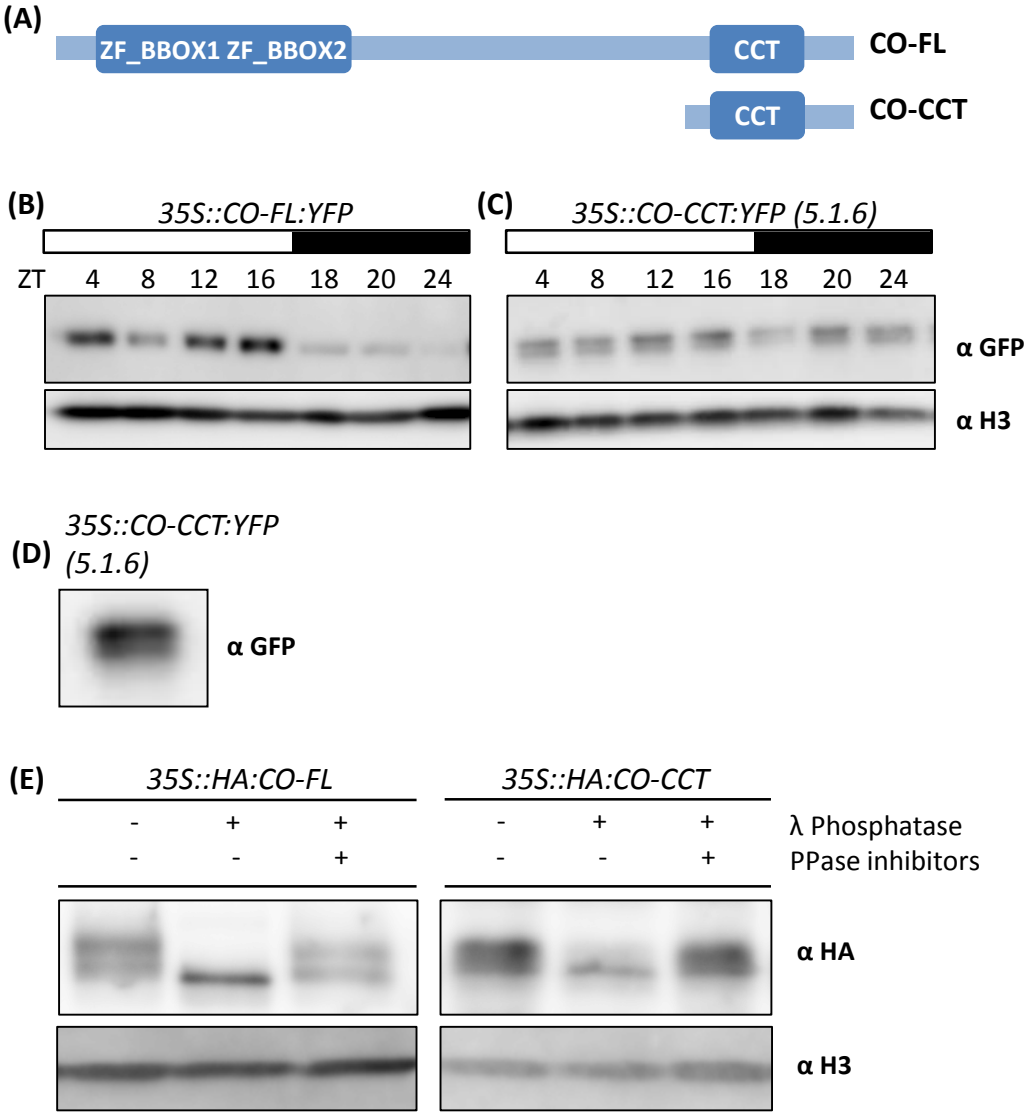
(A)
ZF_BBOX1 ZF_BBOX2
CCT
CO-FL
CCT
CO-CCT
(B)
35S::CO-FL:YFP
ZT 4 8 12 16 18 20 24
(C)
35S::CO-CCT:YFP (5.1.6)
4 8 12 16 18 20 24
α GFP
α H3
(D)
35S::CO-CCT:YFP
(5.1.6)
α GFP
(E)
35S::HA:CO-FL
35S::HA:CO-CCT
- + + - + +
- - + - - +
λ Phosphatase
PPase inhibitors
α HA
α H3

Figure S1

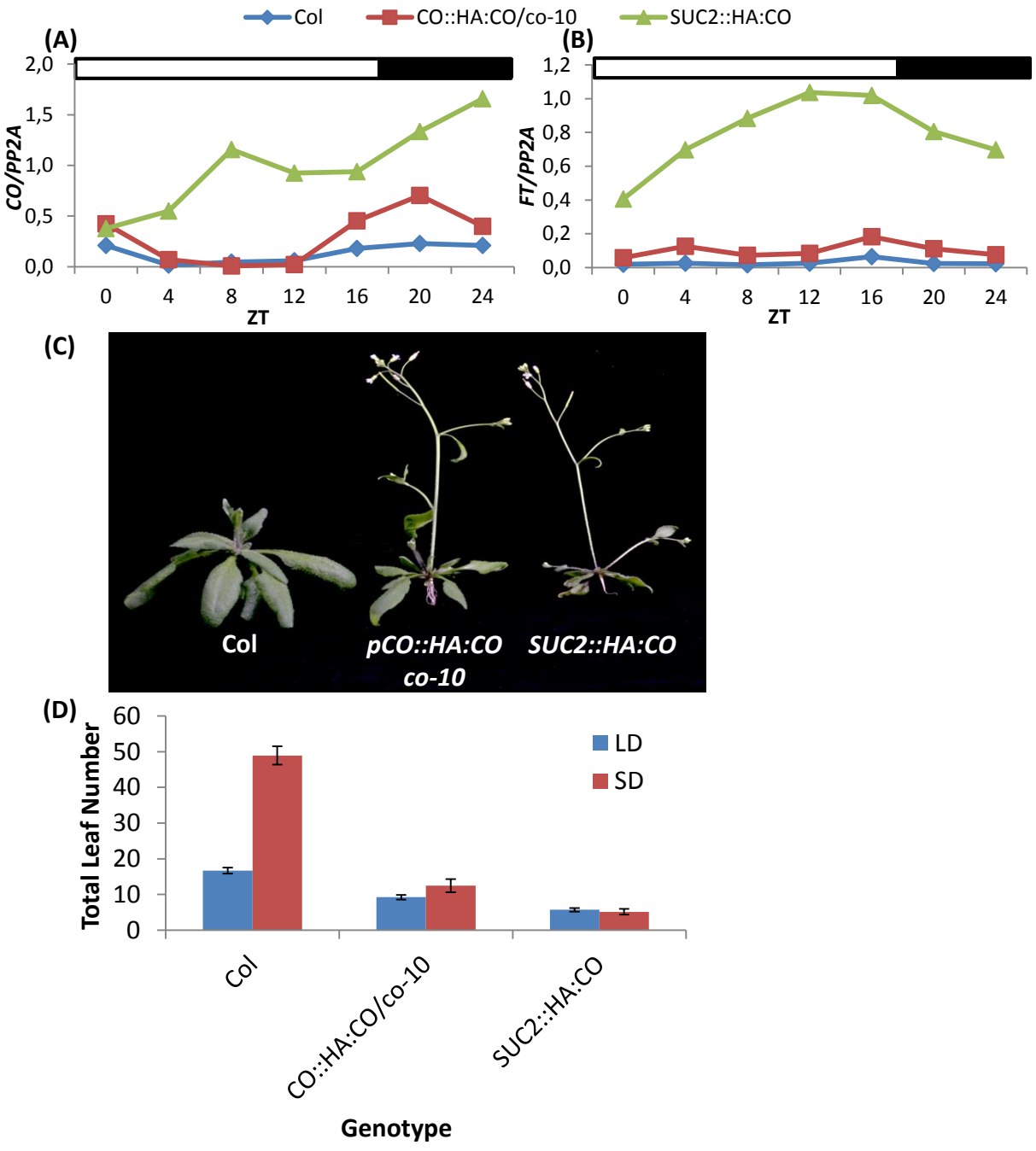
Col
CO::HA:CO/co-10
SUC2::HA:CO
(A)
CO/PP2A
2,0
1,5
1,0
0,5
0,0
0
4
8
12
16
20
24
ZT
(B)
FT/PP2A
1,2
1,0
0,8
0,6
0,4
0,2
0,0
0
4
8
12
16
20
24
ZT
(C)
Col
pCO::HA:CO
co-10
SUC2::HA:CO
(D)
Total Leaf Number
60
50
40
30
20
10
0
LD
SD
Col
CO::HA:CO/co-10
SUC2::HA:CO
Genotype

Figure S2

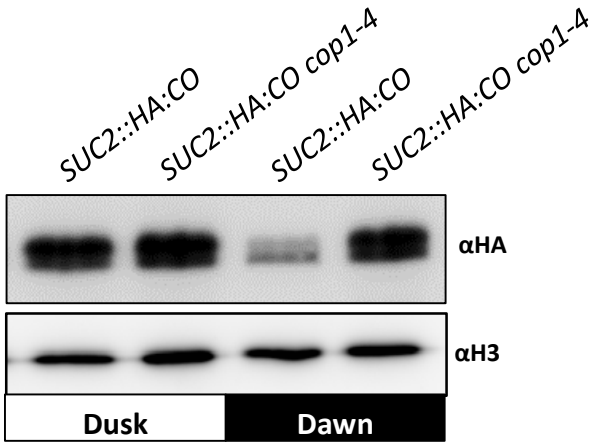
SUC2::HA:CO
SUC2::HA:CO cop1-4
SUC2::HA:CO
SUC2::HA:CO cop1-4
αHA
αH3
Dusk
Dawn

Figure S3

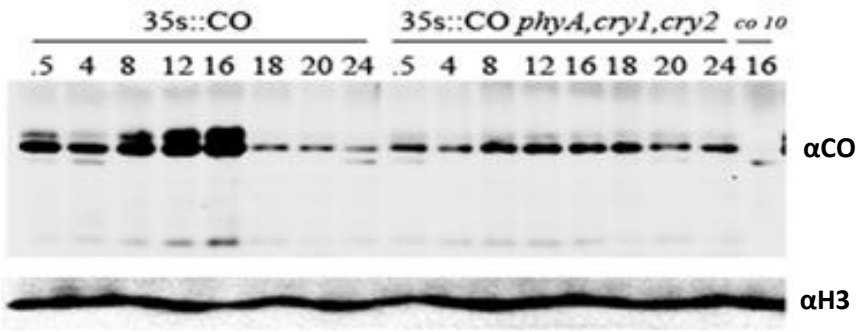
35s::CO
35s::CO phyA,cry1,cry2
co 10
.5 4 8 12 16 18 20 24 .5 4 8 12 16 18 20 24 16
αCO
αH3

Figure S4

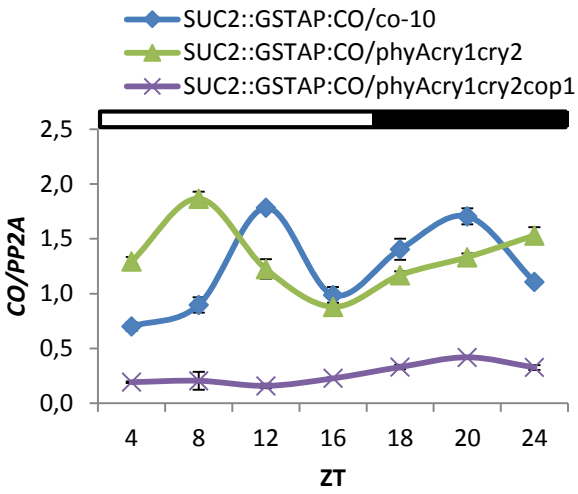
SUC2::GSTAP:CO/co-10
SUC2::GSTAP:CO/phyAcry1cry2
SUC2::GSTAP:CO/phyAcry1cry2cop1
2,5
2,0
1,5
1,0
0,5
0,0
CO/PP2A
4
8
12
16
18
20
24
ZT

Figure S5

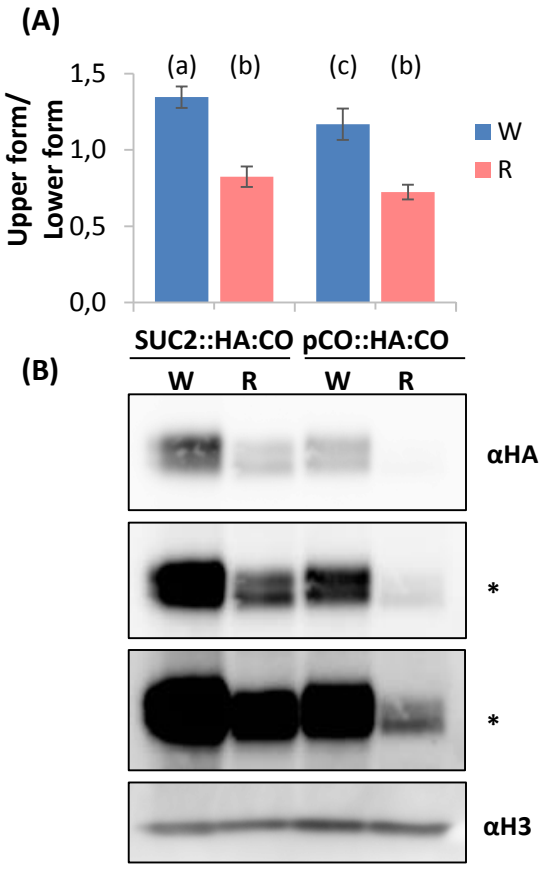
(A)
Upper form/
Lower form
1,5
1,0
0,5
0,0
(a)
(b)
(c)
(b)
W
R
(B)
SUC2::HA:CO
pCO::HA:CO
W
R
W
R
αHA
*
*
αH3

Figure S6