# Photoreceptor Regulation of CONSTANS Protein in Photoperiodic Flowering

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**Supporting Online Material**
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# Photoreceptor Regulation of CONSTANS Protein in Photoperiodic Flowering

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**Many plants flower in response to seasonal fluctuations in day length. The *CONSTANS* (*CO*) gene of *Arabidopsis* promotes flowering in long days. Flowering is induced when *CO* messenger RNA expression coincides with the exposure of plants to light. However, how this promotes CO activity is unknown. We show that light stabilizes nuclear CO protein in the evening, whereas in the morning or in darkness the protein is degraded by the proteasome. Photoreceptors regulate CO stability and act antagonistically to generate daily rhythms in CO abundance. This layer of regulation refines the circadian rhythm in *CO* messenger RNA and is central to the mechanism by which day length controls flowering.**

Day length provides an environmental cue that allows plants to flower in response to the changing seasons (*1*). Long days (LDs) trigger flowering of *Arabidopsis*, and a genetic pathway controlling this response (*2*, *3*) is conserved in distantly related Angiosperms (*4*). In *Arabidopsis*, *CONSTANS* (*CO*) plays a central role in the induction of flowering by LDs and encodes a nuclear protein containing zinc fingers (*5*, *6*). *CO* mRNA abundance is regulated by the circadian clock and accumulates late in the day, when plants growing under LDs are exposed to light (*7*). Under these conditions, CO activates transcription of the *FT* gene, which encodes a RAF-kinase-inhibitor-like protein that promotes flowering (*7*–*11*). Activation of *FT* transcription is proposed to depend on posttranscriptional regulation of CO that is triggered by light, and therefore flowering under LDs occurs because of the coincidence of circadian-clock-controlled transcription of *CO* and light-mediated posttranscriptional regulation (*7*, *8*, *12*). How CO is regulated by light has not been described. We demonstrate that CO protein abundance and activity respond to light and define roles for photoreceptors in controlling these responses.

CO activity can be followed indirectly by measuring *FT* mRNA levels. The *35S::CO* plants, which overexpress *CO* mRNA at constant levels independently of the circadian clock or exposure to light, contain *FT* mRNA at higher abundance during the photoperiod than in the dark (*7*, *8*). A fusion of *FT* regulatory sequences to luciferase (LUC) was introduced into wild-type and *35S::CO* plants and accurately reported the up-regulation of *FT* caused by *CO* overexpression (fig. S1). The *35S::CO FT::LUC* plants were shifted from darkness to light of different wavelengths. Luminescence increased rapidly after exposure to white (W) or blue (B) light and more slowly when plants were exposed to far-red light (FR), but did not markedly increase in red light (R) (Fig. 1A). In *35S::CO* plants, *FT* mRNA abundance showed similar regulation to *FT::LUC*, whereas *CO* mRNA levels were not significantly altered by light (Fig. 1, B and C). Therefore, posttranscriptional regulation of CO activity by B and FR rapidly activates *FT* transcription after transfer from darkness, as described for the maintenance of CO activity after transfer from W (*8*).

We tested whether exposure to light regulates *FT* by influencing the abundance of CO protein. Fluorescence of GFP:CO (green fluorescent protein:CO) was detected in the nucleus of stomatal guard cells of *35S::GFP:CO* plants under W, B, or FR, but not in plants exposed to darkness or R (Fig. 2A). To follow native CO protein, antibodies

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were raised to the central portion of CO, which is most diverged from related proteins. In nuclear extracts of *35S::CO* seedlings, the protein was present at higher abundance after exposure of plants to W or FR and particularly to B, but not to darkness or R (Fig. 2B). CO protein was also detected in nuclear extracts of wild-type plants grown in continuous B (Fig. 2C), but not in those grown in W. As expected, the protein was absent under B in *co-8* mutants, which carry a deletion in *CO* (*6*) (Fig. 2C). The protein therefore accumulates to high levels under B in both wild-type and *35S::CO* plants. Overall, under the light conditions tested, there is a correlation between CO protein levels and the activation of *FT*. The high abundance of CO protein under B may contribute to the extreme early flowering observed under these conditions (*13*, *14*).

We examined whether CO protein abundance correlates with *FT* expression under diurnal cycles of light and dark by following the luminescence of *35S::CO FT::LUC* plants under short days (SDs) and LDs (Fig. 1D). Lower luminescence occurred under SDs than under LDs, and a high amplitude peak 16 hours after dawn under LDs was absent under SDs. RNA extracted from *35S::CO* plants showed a similar diurnal pattern of *FT* regulation (Fig. 1E and fig. S2). However, in *35S::CO FT::LUC*, the high resolution of the luciferase marker detected a peak in luminescence soon after dawn, which was not detected by RNA analysis. Next, the abundance of GFP::CO was tested under LDs and SDs, and GFP fluorescence was detected only when *35S::GFP:CO* plants were exposed to light, and fell rapidly in darkness (Fig. 2D). Similarly, in nuclear extracts of *35S::CO* plants grown under LDs or SDs, CO protein was absent in the dark (Fig. 2E). Under LDs, CO was present at high abundance during the photoperiod only 8, 12, and 16 hours after dawn, and not at 4 hours. Also, under LDs a high amplitude evening peak of CO protein was observed that was absent under SDs (Fig. 2E). Furthermore, a transient peak in CO abundance was detected 30 min after dawn under LDs and SDs that had already declined 30 min later, and this correlated with the early morning peak in FT::LUC expression detected at that time (Fig. 2F). This peak may be due to overexpression of *CO* in *35S::CO* plants, because no peak in *FT* expression has been described at this time in wild-type plants. Overall, the diurnal pattern of *FT::LUC* expression in *35S::CO* plants grown under LDs and SDs closely follows the abundance of CO protein, suggesting that regulation of CO abundance is a major determinant of *FT* expression levels.

We also tested whether CO protein shows diurnal regulation in wild-type plants. CO could not be detected in wild-type plants grown under W; therefore, they were grown under LDs and SDs of B. A high amplitude peak in CO was detected under LDs of B 12 hours and 16 hours after dawn, whereas under SDs the protein was not detected (Fig. 2G). Therefore, under LDs the diurnal rhythm in CO is similar in wild-type and *35S::CO* plants, and its amplitude is reduced in both genotypes under SDs.

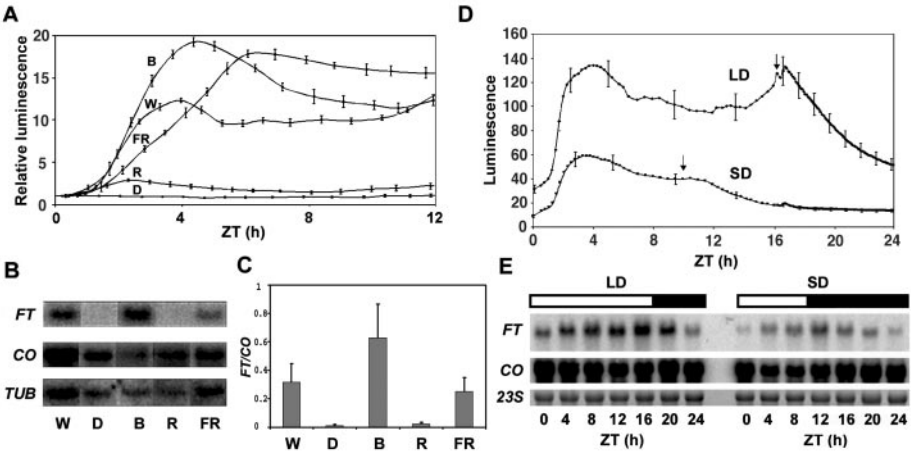


**Fig. 1.** *FT* expression under different light regimes. W, white; D, dark; B, blue; R, red; FR, far-red. (**A**) Luminescence [(counts per second per 10 plants) divided by 10] of *FT::LUC 35S::CO* plants. Luminescence at each time was divided by the level at time 0 to produce relative values. Plants were grown in the dark for 2 days before transfer to different light conditions. Data are the mean with standard error for 10 plants. (**B**) *FT* and *CO* mRNA in *35S::CO* plants analyzed on Northern blots. Plants were treated as in A and kept for 2 days in continuous light prior to RNA extraction. *TUBULIN* (*TUB*) was used as a loading control. (**C**) Mean with standard error of three experiments as shown in (B). (**D**) Luminescence [(counts per second per 10 plants) divided by 10] of *FT::LUC 35S::CO* plants during 24 hours under LDs or SDs. Arrows indicate when lights were turned off. Data are the mean for 96 plants; standard error is shown for each tenth data point. (**E**) *CO* and *FT* mRNA in *35S::CO* plants during 24 hours under LDs or SDs analyzed by Northern blotting. *23S* RNA was the loading control. Plants used in (A) to (E) were 7 days old. ZT, Zeitgeber time.

Degradation by the 20S proteasome regulates the abundance of certain transcription factors in response to light/dark transitions (*15*, *16*). To determine the involvement of the proteasome in CO regulation, LD-grown seedlings were treated with proteasome inhibitors. These inhibitors stabilized CO during the morning and in darkness (Fig. 3A). Ubiquitination of CO was tested in vitro by adding recombinant CO to nuclear extracts of seedlings grown in light or dark, as described previously (*15*) (Fig. 3B). After the addition of adenosine triphosphate (ATP), the protein was degraded in the extract. In the presence of proteasome inhibitors, CO antibody predominantly detected larger molecular weight forms. Proteins of similar size were also detected by antibody to ubiquitin. These forms were not detected in extracts lacking recombinant CO, suggesting that they represent ubiquitinated forms of CO. The results of the in vivo and in vitro experiments suggest that CO is ubiquitinated and degraded by the proteasome.

Phytochrome (phy) and cryptochrome (cry) photoreceptors regulate flowering and were proposed to do so through CO (*8*, *14*, *17*, *18*), although only phyA was shown to affect *CO* mRNA abundance (*19*). To identify the photoreceptors responsible for the posttranscriptional activation of *CO*, *FT* mRNA abundance was tested in *35S::CO* plants carrying photoreceptor mutations (Fig. 4A). These experiments indicated that *CO* function is enhanced by posttranscriptional regulation through the photoreceptors cry1, cry2, and phyA, which is in agreement with *FT* expression levels in otherwise wild-type plants carrying photoreceptor mutations (*8*, *20*, *21*).

The effects of these photoreceptors and of phyB on the abundance of CO protein were tested in *35S::CO* plants. The *phyB* mutation caused an increase in CO protein abundance in R (Fig. 4B) and early in the photoperiod (Fig. 4C). Thus, phyB reduces CO abundance in R and during the morning, which is consistent with the higher expression of *FT* detected in *phyB* mutants (*20*, *21*). The *cry1 cry2* mutations markedly reduced the level of CO protein under B (Fig. 4B and fig. S3), and the peaks in CO abundance during the morning and evening (Fig. 4D), demonstrating that cryptochromes stabilize CO at these times. Therefore, under LDs the cryptochromes antagonize the degradation of CO mediated by phyB and stabilize CO under B. Flowering-time measurements suggested that cry2 promotes flowering by antagonizing the repressive effect of phyB (*14*), and our data indicate that CO abundance is a major target of this antagonism. However, because the *cry1 cry2* mutations dramatically reduce *FT*

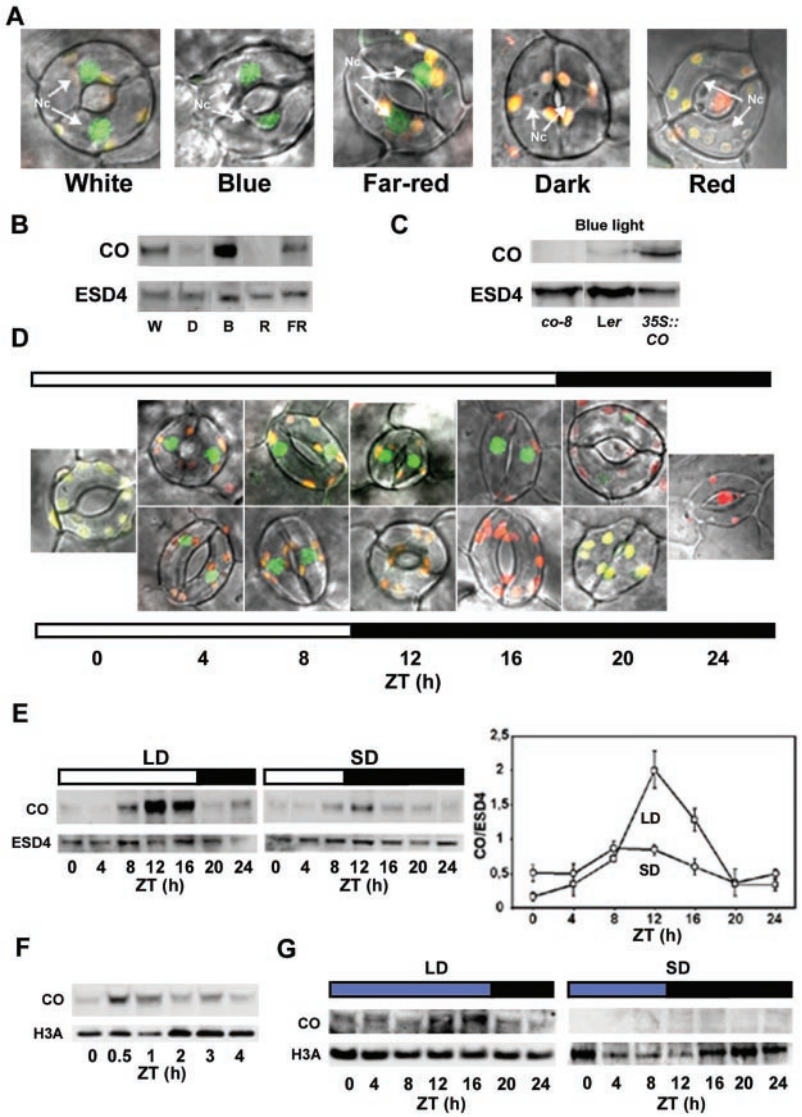


**Fig. 2.** CO protein abundance under different light conditions. (**A**) Confocal images of guard cells of *35S::GFP:CO* plants exposed for 2 days to different light conditions. Green fluorescence represents GFP:CO; the red-yellow signal is plastid autofluorescence. Arrows indicate nuclei (Nc). (**B**) CO protein in nuclear extracts of *35S::CO* plants after a 2-day light treatment analyzed by Western blotting. Early in short days 4 (ESD4) (*24*) used as a loading control. (**C**) CO protein in nuclear extracts of *co-8*, wild-type L*er*, and *35S::CO* plants exposed for 2 days to blue light. Detection and control as for (B). (**D**) Confocal images of guard cells of *35S::GFP:CO* plants during 24 hours under LDs (top) or SDs (bottom) of white light. (**E**) CO protein in nuclear extracts of *35S::CO* plants during 24 hours under LDs (left) or SDs (right). Detection and control as for (B). Graph shows quantification of CO protein abundance as the mean of three LD and two SD experiments. (**F**) CO protein in nuclei of *35S::CO* plants grown under LDs and harvested in the morning. CO analyzed by Western blotting. Antibody to Histone 3A (H3A) used as a control. (**G**) CO protein in nuclear extracts of wild-type (L*er*) plants grown under LDs or SDs of blue light. CO and control detected as in (F). ZT, Zeitgeber time.

mRNA levels under B but have a lesser effect on CO protein abundance (Fig. 4, A and B), they are likely to have an additional role in activating CO after its accumulation. In FR, phyA stabilizes CO protein, and in LDs it has a similar role to the cryptochromes in stabilizing CO during the photoperiod (Fig. 4, B to D; fig. S3). Finally, the *phyA* and *cry1 cry2* mutations delay flowering of *35S::CO* plants whereas the *phyB* mutation accelerates flowering (table S1), consistent with the effects of these mutations on CO protein abundance and *FT* expression.

The circadian rhythm in *CO* mRNA abundance was proposed to represent a photoperiod response rhythm, in which exposure to light coincides with *CO* expression only under LDs and thereby triggers flowering (*7*, *8*). We provide a mechanism for the effect of light by showing that CO protein is stabilized at the end of a LD when the abundance of *CO* mRNA peaks in wild-type plants (Fig. 4E). This mechanism is independent of transcrip-

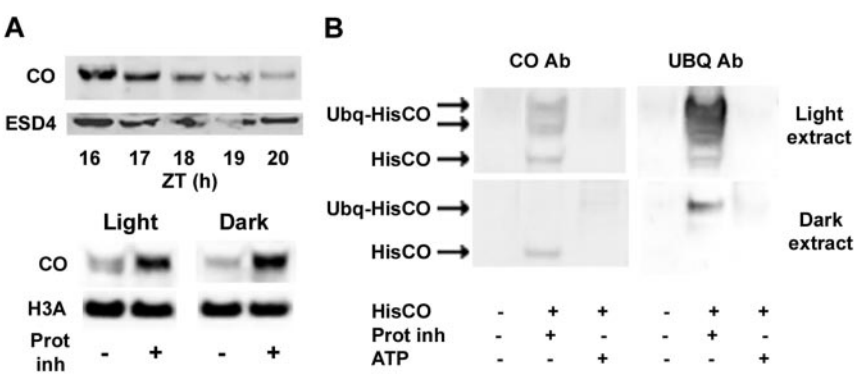


**Fig. 3.** Effect of the proteasome on CO stability. (**A**) CO protein in nuclear extracts of LD-grown *35S::CO* plants during the first 4 hours of the dark period (upper panel). CO protein in nuclear extracts of *35S::CO* plants after incubation for 4 hours with proteasome inhibitors (lower panel). Protein extracted 3 hours (light, left panel) and 19 hours (dark, right panel) after dawn. Antibody to H3A or to ESD4 used to detect loading controls. (**B**) In vitro assay to detect ubiquitinated CO. HisCO protein (200 ng) was added to cell-free extracts of L*er* plants grown for 2 days under continuous light (upper panels) or dark (lower panels). CO (left) or proteins modified by ubiquitin (UBQ) (right) were detected by Western blotting. Either proteasome inhibitors (*24*) or ATP were added. ZT, Zeitgeber time.

tion and represents a second layer of regulation in which photoreceptors drive diurnal rhythms in CO protein abundance. We propose that early in the day phyB promotes the degradation of CO (Fig. 4E), whereas in the evening cryptochromes and phyA antagonize this degradation and stabilize CO protein, allowing the activation of *FT* (Fig. 4E). How the balance of activity between phyB and cryptochromes changes between the morning and the evening is not clear, especially because phyB is light stable and the cryptochromes are present constantly throughout a LD (*22*, *23*). However, the lower abundance of cry2 in SDs (*22*, *23*) may explain why CO activates *FT* less strongly under these conditions. The degradation of CO in the dark may be stimulated by dark-dependent ubiquitin ligases (Fig. 4E) related to those previously described (*15*, *16*). These posttranscriptional effects ensure that CO protein accumulates only at the end of a LD, whereas *CO* mRNA is present more extensively. This combination of regulatory mechanisms may allow the plant to respond to lengthening days, because the peak in CO protein may broaden as the photoperiod lengthens and provide a quantitative flowering response to longer photoperiods. Increased CO protein abundance may also induce early flowering in response to the low R:FR ratios to which plants are exposed when shaded by neighboring vegetation. Reduced phyB activity associated with shade avoidance would increase CO protein abundance and contribute to the increased *FT* expression previously observed (*20*, *21*).

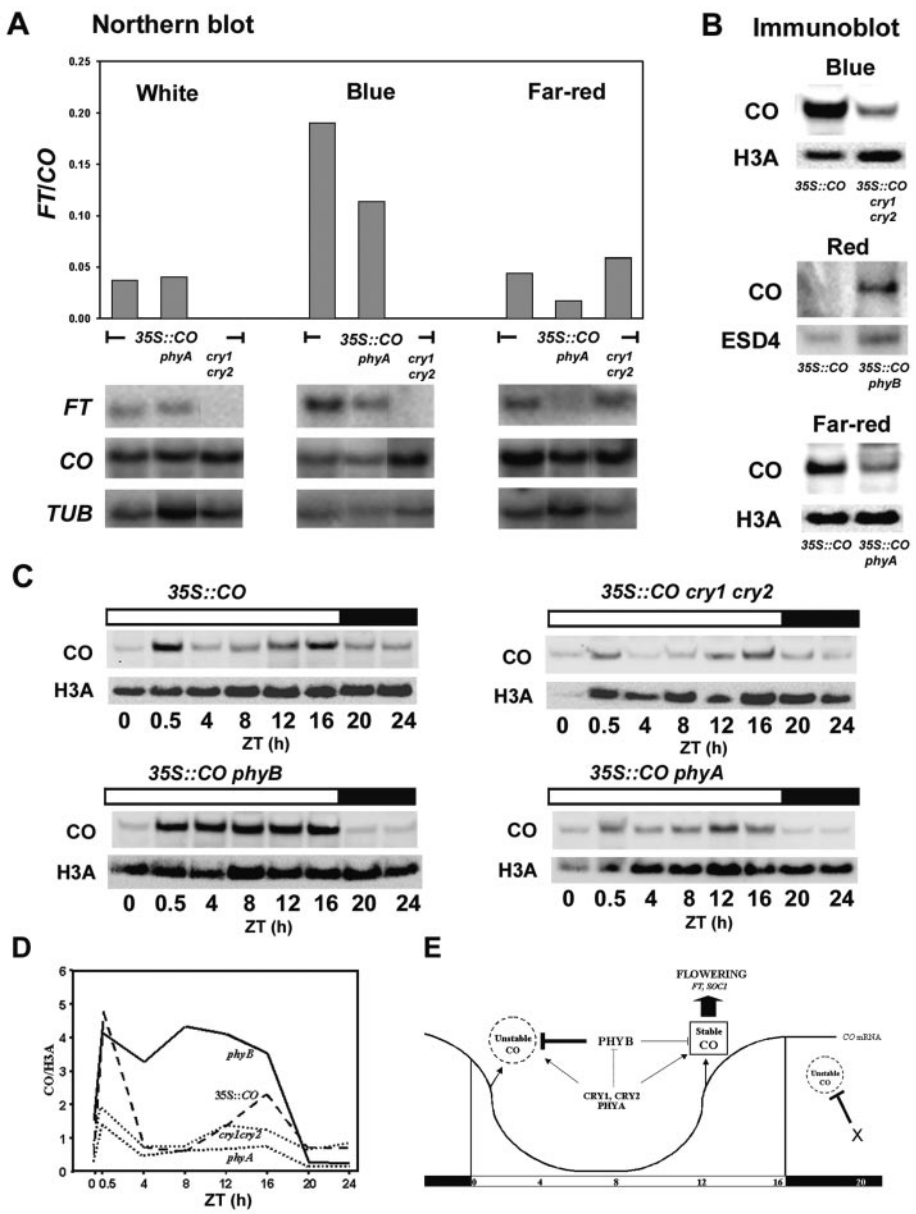


**Fig. 4.** Effect of photoreceptor mutations on CO activity and stability. (**A**) *FT* mRNA detected by Northern blotting. The 35S::*CO* plants carrying photoreceptor mutations were grown for 7 LDs and 2 additional days in continuous W, B, or FR. Northern blots probed with *FT*, *CO*, and *TUB* probes. (**B**) CO protein detected in nuclear extracts of 35S::*CO* plants carrying photoreceptor mutations. Growth conditions as for (A). Western blots were probed with CO, H3A, or ESD4 antibodies. (**C**) CO protein in nuclear extracts of *35S*::CO plants carrying photoreceptor mutations during a 24-hour LD cycle. Western blots probed with CO or H3A antibodies. (**D**) CO protein abundance compared with H3A in a similar time course to (C). (**E**) Model of CO protein regulation related to mRNA levels in wild-type plants. Arrows indicate stabilization/activation of the protein, and perpendicular lines illustrate degradation/inactivation. CO protein activity is restricted to the end of a LD. ZT, Zeitgeber time.

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