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# High-level expression and phosphorylation of phytochrome B modulates flowering time in Arabidopsis

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## SUMMARY

**Optimal timing of flowering in higher plants is crucial for successful reproduction and is coordinated by external and internal factors, including light and the circadian clock. In *Arabidopsis,* light-dependent stabilization of the rhythmically expressed CONSTANS (CO) is required for the activation of *FLOWERING LOCUS T* (*FT*), resulting in the initiation of flowering. Phytochrome A and cryptochrome photoreceptors stabilize CO in the evening by attenuating the activity of the CONSTITUTIVE PHOTOMORPHOGENIC 1–SUPPRESSOR OF PHYA-105 1 (COP1–SPA1) ubiquitin ligase complex, which promotes turnover of CO. In contrast, phytochrome B (phyB) facilitates degradation of CO in the morning and delays flowering. Accordingly, flowering is accelerated in *phyB* mutants. Paradoxically, plants overexpressing phyB also show early flowering, which may arise from an early phase of rhythmic *CO* expression. Here we demonstrate that overexpression of phyB induces *FT* transcription at dusk and in the night without affecting the phase or level of *CO* transcription. This response depends on the light-activated Pfr form of phyB that inhibits the function of the COP1–SPA1 complex by direct interactions. Our data suggest that attenuation of COP1 activity results in the accumulation of CO protein and subsequent induction of *FT*. We show that phosphorylation of Ser-86 inhibits this function of phyB by accelerating dark reversion and thus depletion of Pfr forms in the night. Our results explain the early flowering phenotype of phyB overexpression and reveal additional features of the molecular machinery by which photoreceptors mediate photoperiodism.**

**Keywords: flowering, phytochrome, circadian clock, COP1, CONSTANS, *Arabidopsis thaliana*.**

## INTRODUCTION

Flowering is regulated not only by developmental signals, but also by environmental cues like day length, quality of light or abiotic stress. Many plants use day length as an indicator of the actual season of the year, to be preferred or avoided as the time to set seeds. Arabidopsis is a facultative long-day plant, meaning that flowering is initiated much earlier under long-day (LD) conditions (e.g. 16 h light/8 h dark cycles) than under short-day (SD) conditions (e.g. 8 h light/16 h dark cycles). Photoperiodic time measurement in Arabidopsis is based on the functional interaction of the endogenous circadian clock and environmental light signals mediated by photoreceptors.

Circadian clocks are biochemical timing mechanisms that temporarily modulate the function of several signaling (light, hormonal, stress) pathways by controlling the expression of key components according to a daily rhythm with 24 h period (Covington *et al.*, 2008; Hsu and Harmer, 2014). The prevailing influence of the clock on plant physiology is indicated by the fact that 30–40% of the expressed genes are rhythmically regulated (Covington *et al.*, 2008). The most apparent adaptive advantage of circadian clocks is the precise temporal organization of cellular processes within the day. To fulfill this role, the clock must be synchronized to the day/night cycle via daily environmental cues, like temperature and light.

Phytochromes (phyA–E in Arabidopsis) are red/far-red light absorbing chromoproteins with a covalently bound chromophore (Franklin and Quail, 2010). phyA and phyB

are the most abundant and important members of the family. In the dark, phytochromes are present in the inactive red light absorbing form (Pr), which is converted to the active far-red light absorbing conformer (Pfr) upon red light irradiation. The Pfr form is promptly converted back to the Pr form by absorbing far-red light (photoconversion), or by a slower, light-independent process called dark reversion (Rockwell *et al.*, 2006). Phosphorylation of phyB at Ser-86 accelerates dark reversion of the receptor (Medzihradszky *et al.*, 2013). Plants overexpressing the non-phosphorylatable (Ser86Ala substitution) or the phospho-mimic (Ser86Asp substitution) mutant derivatives of phyB showed increased or reduced light responses respectively, mainly at low fluences of red light (low Pr to Pfr photoconversion rate) or under simulated shade (high Pfr to Pr photoconversion rate), where Pfr levels are limited (Medzihradszky *et al.*, 2013). Cryptochromes (CRY1 and CRY2) are flavin-binding chromoproteins absorbing blue light (Chaves *et al.*, 2011). The family of LOV domain F-box proteins consists of ZEITLUPE (ZTL), FLAVIN BINDING, KELCH REPEAT, F-BOX (FKF1) and LOV KELCH PROTEIN 2 (LKP2) (Ito *et al.*, 2012). These proteins absorb blue light and are functional constituents of Skp1/Cullin/F-box (SCF) type E3 ubiquitin ligase complexes involved in light-dependent destabilization of proteins associated with the circadian clock and the photoperiodic induction of flowering (Ito *et al.*, 2012).

In Arabidopsis, transcription and protein stability of the zinc-finger B-box type transcription factor *CONSTANS* (*CO*) are regulated by the clock, ubiquitin ligases and photoreceptors in a way that the high level of CO proteins is restricted to the evenings of long days (Yanovsky and Kay, 2002; Valverde *et al.*, 2004). CO directly activates the expression of *FLOWERING LOCUS T* (*FT*), which in turn triggers flowering (Samach *et al.*, 2000). The clock components CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), two related Myb transcription factors, drive the expression of CYCLING DOF FACTORs, which redundantly and directly repress *CO* transcription in the morning (Nakamichi *et al.*, 2007). In the afternoon and the evening, FKF1 forms a complex with the clock protein GIGANTEA (GI) that degrades CDF proteins allowing *CO* transcription to rise (Sawa *et al.*, 2007). CO protein turnover is regulated by ubiquitin ligases and photoreceptors. HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1 (HOS 1) is a Ring-finger E3 ubiquitin ligase that destabilizes CO during the day (Lazaro *et al.*, 2012). ZTL promotes turnover of CO in the morning and a similar function of LKP2 was suggested (Song *et al.*, 2014). In contrast, FKF1 stabilizes CO by direct interaction in the afternoon (Song *et al.*, 2012). TARGET OF EAT1 (TOE1) binds to FKF1 and indirectly destabilizes CO in the afternoon (Zhang *et al.*, 2015). TOE1 also binds to CO in the morning and prevents CO-mediated induction of *FT.* In the evening, but particularly during the night, the E3 ubiquitin ligase CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) destabilizes CO. COP1 functions in a complex with the SUPPRESSOR OF PHYA-105 1–4 (SPA1–4) proteins, which are required for efficient ubiquitination (Zhu *et al.*, 2008). CRY1 and CRY2 interact with SPA1 in a blue-light-dependent manner that results in the inhibition of COP1 activity and the accumulation of CO protein in the evening of long days (Lian *et al.*, 2011; Zuo *et al.*, 2011). phyA also stabilizes CO at this time in a light-dependent manner (Valverde *et al.*, 2004) that may involve deactivation of COP1 by phyA–SPA1 interaction (Sheerin *et al.*, 2015). In contrast, phyB promotes degradation of CO in the first half of the day in a red-light-dependent manner (Valverde *et al.*, 2004). PHYTOCHROME-DEPENDENT LATE FLOWERING (PHL) interacts with both phyB Pfr and CO and appears to shield CO from the effects of phyB, thus contributing to accumulation of CO in the evening (Endo *et al.*, 2013).

Overexpression of phyB results in early flowering especially under SD conditions that is in sharp contrast with the above described role of the receptor (Bagnall *et al.*, 1995). The primary aim of our work was to solve this paradox by revealing the molecular mechanism by which phyB overexpression accelerates flowering. We show that early flowering of phyB overexpressors is not due to altered clock function. By means of physiological tests and analysis of genetic interactions we demonstrate that phyB overexpression acts through the CO-FT regulon to promote flowering. We show that *FT* expression is significantly induced in the evening and in the night in phyB-overexpressing lines. Using phyB mutants with conditionally or constitutively altered levels of the active form of phyB we show that induction of *FT* requires phyB Pfr. Finally, we present data suggesting that inactivation of COP1 by the phyB–SPA1 interaction leads to the accumulation of CO protein and subsequent induction of *FT* gene expression.

## RESULTS

### Early flowering phenotype of phyB-overexpressing plants is not caused by altered circadian rhythms

The well characterized transgenic line expressing the phyB–GFP fusion protein under the control of the CaMV 35S promoter in the *phyB-9* background (phyB[WT]) was used to analyze the effect of phyB overexpression on the induction of flowering (Medzihradszky *et al.*, 2013). As we hypothesized that the level of phyB Pfr is crucial for the flowering response, we also included plants overexpressing mutant derivatives of phyB that block or mimic phosphorylation at Ser-86 (phyB[S86A] or phyB[S86D], respectively) affecting dark reversion rate and, depending on light conditions, Pfr levels (Medzihradszky *et al.*, 2013). Plants were grown under short-day (8 h light/16 h dark, SDs) or under long-day (16 h light/8 h dark, LDs)

conditions and flowering time was determined. In agreement with previous results (Bagnall *et al.*, 1995; Endo *et al.*, 2005), both *phyB-9* and phyB[WT] plants flowered earlier than the wild-type Columbia (Col) plants (Figure 1a–c). The phenotype was more pronounced in SD, but flowering of both lines retained sensitivity to photoperiods. All phyB overexpressors flowered at the same time in LDs, but flowering of phyB[S86A] was early and phyB [S86D] was late compared to that of phyB[WT] in SD. The very similar level of phyB derivatives excluded different expression as explanation of the phenotypes (Figure S1a).

Stabilization of CONSTANS (CO) protein during the day is thought to account for accelerated flowering of *phyB* mutants (Valverde *et al.*, 2004), but the mechanism underlying the early flowering phenotype of phyB overexpressors is not known. Overexpression of phyB is expected to shorten the period of the circadian clock in the light (Hall *et al.*, 2002) that could cause early phasing of circadian rhythms. According to the external coincidence model, such a phase change may result in stabilization of CO and acceleration of flowering especially in SDs (Yanovsky and Kay, 2002). To test this hypothesis, expression of *GI* was

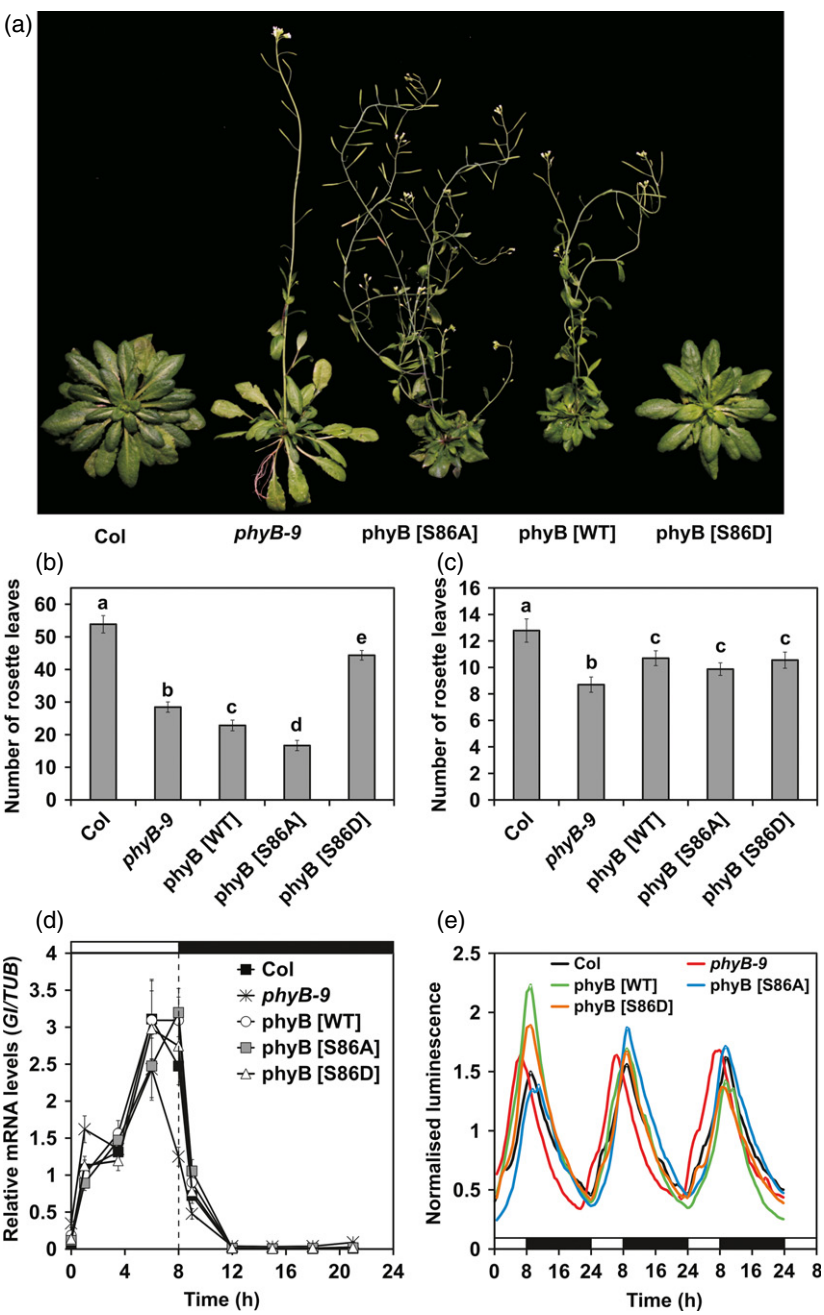


**Figure 1.** The early flowering phenotype of phyB overexpressors is not caused by alteration of the circadian clock.
(a) Images of representative Col, *phyB-9*, phyB[WT], phyB[S86A] and phyB[S86D] plants grown under short-day (SD) conditions. Plants were grown in 8 h light/16 h dark photocycles. Images were taken 45 days after sowing.
(b) Flowering time under SD conditions. Plants were grown under 8 h light/16 h dark photocycles. Error bars indicate standard error (SE), and different letters show significant differences at $P < 0.01$ (Duncan's test).
(c) Flowering time under long-day (LD) conditions. Plants were grown in 16 h light/8 h dark photocycles. Error bars indicate SE, and different letters show significant differences at $P < 0.05$ (Duncan's test).
(d) *GI* mRNA levels under SD conditions. Plants were grown in 8 h light/16 h dark photocycles for 10 days. *GI* and *TUB* mRNA levels were determined by qPCR assays. *GI* values normalized to the corresponding *TUB* values are plotted. White and black bars indicate light and dark conditions, respectively. Error bars represent SE.
(e) *GI:LUC* rhythms under SD conditions. Plants expressing *GI:LUC* were grown in 8 h light/16 h dark photocycles for 10 days. Luminescence was monitored for days 8–10. For each line, luminescence values were normalized to the average of counts recorded during the assay.

monitored in SDs. GI is a component of the circadian oscillator and is considered as a key factor regulating rhythmic expression of *CO* (Locke *et al.*, 2005; Fornara *et al.*, 2009). Accumulation of *GI* mRNA was not affected by phyB overexpression in SDs, but showed slightly early phase in *phyB-9* (Figure 1d) as reported previously (Salome *et al.*, 2002). To determine the phase of *GI* expression more precisely, the *GI:LUC* reporter was introduced in the lines. *phyB-9* plants showed early phase of *GI:LUC* expression, but no changes were detected for any of the overexpression lines (Figure 1e). These results demonstrate that: (i) overexpression of phyB causes an early flowering phenotype especially in SDs; (ii) this function of phyB is modulated by phosphorylation at Ser-86; and (iii) the phase of the circadian oscillator was not altered in the lines overexpressing phyB.

### Phosphorylation of phyB modulates red light signaling to the clock

Since the phase of the clock was not influenced by phyB overexpression in white light/dark cycles (Figure 1d,e), the function of red light input to the clock was tested by more specific assays in these lines. In plants, the free-running period shortens with increasing fluence rate of continuous light (Aschoff's rule, parametric entrainment). To test this response, plants expressing the *CCA1:LUC* reporter were assayed in continuous red light at different fluence rates, and periods were estimated (Figure 2a). Col plants showed the expected marked period change in response to light intensity, whereas *phyB-9* plants produced longer periods almost throughout the fluence rate range. phyB[WT] plants had shorter periods as compared with Col at lower fluences of red light. phyB[S86A] plants showed even stronger response, as having shorter periods than that of phyB [WT] and failing to respond to changes in fluence rate in the 10–75 μmol $m^{-2}$ $s^{-1}$ range. In contrast, phyB[S86D] plants produced periods indistinguishable from those of *phyB-9* at lower and medium fluences, but at high fluence rates (50–75 μmol $m^{-2}$ $s^{-1}$) they were very similar to Col, phyB[WT] and phyB[S86A] plants. However, no period differences were detected among the control and phyB-overexpressing lines, when they were assayed in constant darkness or continuous blue light (Figure 2b). These data indicate that phosphorylation of phyB at Ser-86 strongly inhibits the function of the receptor in parametric entrainment at low and medium fluence rates (1–35 μmol $m^{-2}$ $s^{-1}$) of continuous red light. The lack of period phenotype of phyB[S86D] at higher fluence rates is reminiscent of the hypocotyl elongation response of these plants (Medzihradszky *et al.*, 2013). These findings are explained by the combined effects of overexpression and the high rate of photoconversion under these conditions, leading to saturating levels of phyB Pfr that are not affected significantly even by accelerated dark reversion. The circadian oscillator free-running in darkness responds to discrete light pulses with characteristic phase shifts (non-parametric entrainment) (Kevei *et al.*, 2007). The

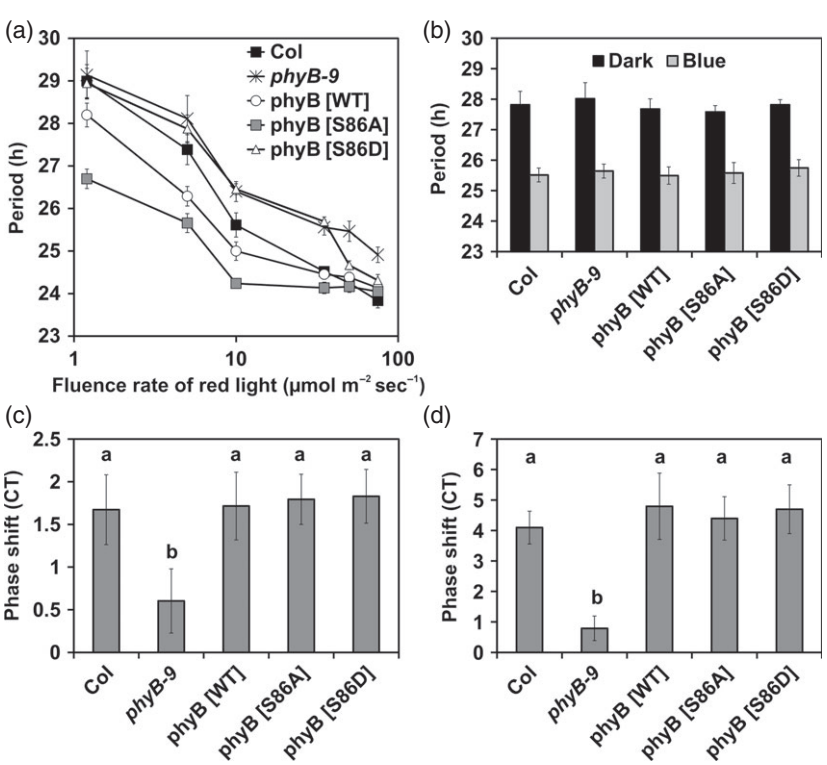


**Figure 2.** Effect of phyB phosphorylation on red light input to the clock.
(a) Red light fluence rate response curves. Plants expressing *CCA1:LUC* were grown in 12 h light/12 h dark photocycles for 7 days and transferred to different fluence rates of continuous red light. The period of luminescence rhythms were determined and plotted as a function of fluence rate. Error bars represent standard error (SE).
(b) *CCA1:LUC* periods in continuous dark or blue light conditions. Plants expressing *CCA1:LUC* were grown in12 h light/12 h dark photocycles 7 days and transferred to dark or blue light at 10 μmol $m^{-2}$ $s^{-1}$ fluence rate. Data of the first 48 h after the transfer were omitted from period estimation. Error bars represent SE.
(c, d) Red-light-induced phase shifts. Plants expressing *CCA1:LUC* were grown in 12 h light/12 h dark photocycles for 7 days and transferred to darkness. Luminescence was monitored at 1-h intervals after the transfer for 5 days; 33 h after the transfer the plants were treated with a 1 h red light pulse at 1 (c) or 75 (d) μmol $m^{-2}$ $s^{-1}$ fluence rate. Light-induced phase advances converted to circadian time (CT) are shown. Error bars indicate SE, and different letters show significant differences at $P < 0.01$ (Duncan's test).

magnitude of the shifts depends on the dose of the light pulse. To test this response, 7-day-old plants expressing the *CCA1:LUC* reporter were transferred and assayed in darkness. After 33 h in darkness, separate groups of plants were illuminated with red light at 1 μmol $m^{-2}$ $s^{-1}$ (Figure 2c) or 75 μmol $m^{-2}$ $s^{-1}$ (Figure 2d) fluence rate for 1 h, returned to darkness and the measurement was resumed. Phase shifts were calculated by comparing the phase of the second peak after the time of light pulse in the induced versus the non-induced plants. All lines showed phase advances (i.e. phase shifts with positive values). *phyB-9* plants produced weak phase shifts (0.6–0.7 h) that were not affected by the dose of the light pulse. Col and the phyB-overexpressing lines showed no differences in responding to light pulses. These lines produced stronger phase shifts than *phyB-9* plants at both treatments and the magnitude of shifts showed about two-fold increase between the low and high dose light treatments (1.6–1.9 h versus 4.3–4.8 h, respectively). These results suggest that endogenous levels of phyB are required and sufficient for dose-dependent red light-mediated resetting of the clock.

### The early flowering phenotype and elevated levels of *FT* transcripts in phyB-overexpressing lines depends on CO

To reveal the molecular background of the early flowering phenotype, *CO* and *FT* mRNA levels were determined in plants grown under SDs (Figure 3a,b) or LDs (Figure 3c,d). The pattern and level of *CO* expression were not significantly different among the lines tested, except for *phyB-9* plants, which showed early phase of *CO* mRNA accumula-

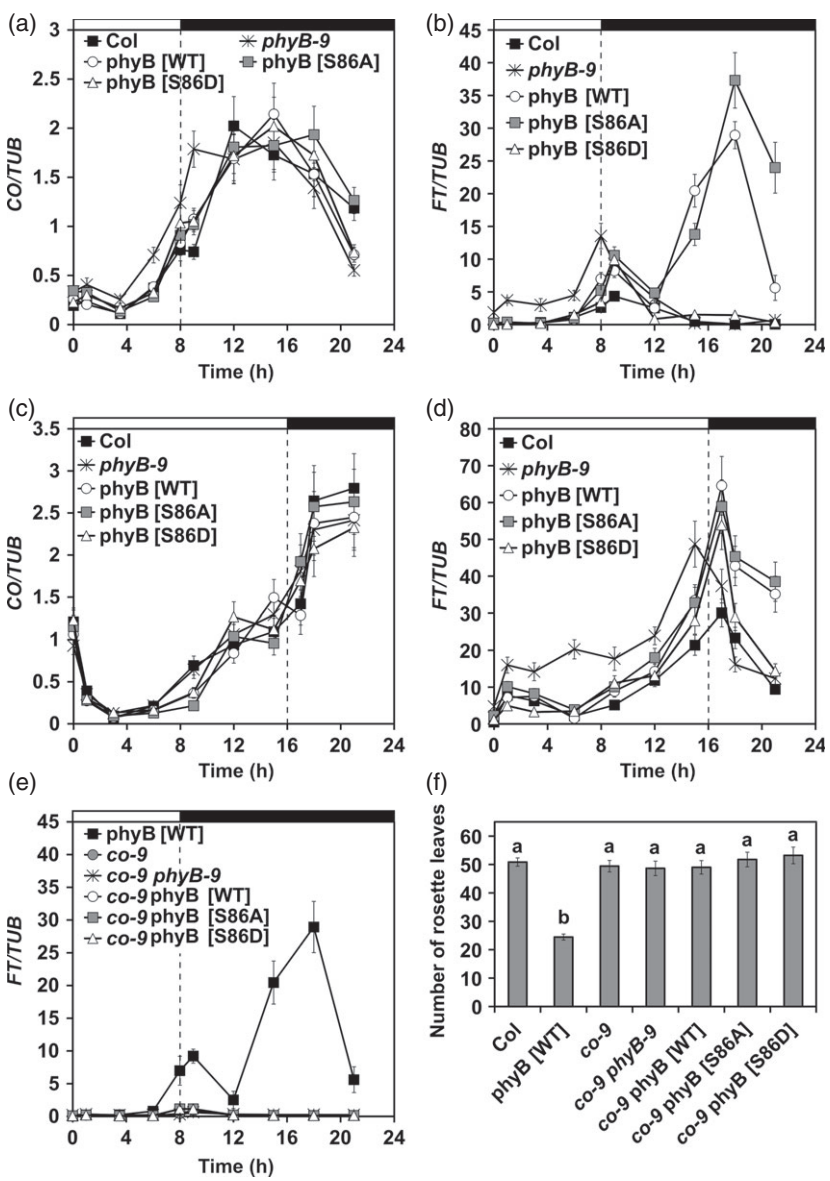


**Figure 3.** The early flowering phenotype depends on FT and CO.
(a, b, e) *CO* and *FT* mRNA levels under short-day (SD) conditions. Plants were grown under 8 h light/16 h dark photocycles for 10 days. *CO, FT* and *TUB* mRNA levels were determined by qPCR assays. *CO* (a) and *FT* (b) and (e) values normalized to the corresponding *TUB* values are plotted. White and black bars indicate light and dark conditions, respectively. Error bars represent standard error (SE).
(c, d) *CO* and *FT* mRNA levels in LD conditions. Plants were grown under 16 h light/8 h dark photocycles for 10 days. *CO, FT* and *TUB* mRNA levels were determined by qPCR assays. *CO* (c) and *FT* (d) values normalized to the corresponding *TUB* values are plotted. White and black bars indicate light and dark conditions, respectively. Error bars represent SE.
(f) Flowering time under SD conditions. Plants were grown under 8 h light/16 h dark photocycles. Error bars indicate SE; $^{a,b}$different letters show significant differences at $P < 0.01$ (Duncan's test).

tion specifically in SD (Figure 3a). These data ultimately demonstrate that the early flowering phenotype of phyB overexpressors is not a consequence of phase alterations of the circadian clock, but probably represent a more direct effect of phyB on one or more components of the photoperiodic pathway. In Col plants *FT* showed the peak of expression around the light-to-dark transition in both photoperiods, but overall *FT* levels were much lower in SDs than in LDs (Figure 3b,d). *FT* expression in *phyB-9* plants was elevated throughout the day, but dropped rapidly to the level of wild-type after lights-off. This observation is in agreement with the role of phyB in the promotion of CO degradation in the light (Valverde *et al.*, 2004). In SDs (Figure 3b), phyB-overexpressing lines accumulated *FT* mRNA at wild-type levels during the day and a two-fold increase was detected as compared with Col just after dusk. The peak was followed by a rapid decline, and *FT* levels in phyB[S86D] plants stayed low during the night, just as in Col or *phyB-9*. In contrast, *FT* mRNA levels in phyB[WT] and phyB[S86A] plants showed dramatic increase in the night peaking around ZT18. In LDs, *FT* mRNA levels in the phyB-overexpressing lines were very similar during the day, but higher around dusk compared with Col. Following the peak, *FT* levels in phyB[S86D] returned to the level of the wild-type, but remained high in phyB[WT] and phyB [S86A] plants. These data demonstrate that overexpression of phyB up-regulates *FT* transcription in the night and that this function of phyB is attenuated by phosphorylation at Ser-86. We showed that this regulation is not due to diurnal changes in the abundance of overexpressed phyB proteins (Figure S1b). Importantly, these results also indicate that phyB overexpression does not induce *FT* transcription during the day, but causes an increase in *FT* levels around dusk, and this effect of phyB is independent of phosphorylation at Ser-86. The tight correlation between *FT* levels and flowering time (Figures 1a,b and 3a–d) suggested that elevated *FT* expression is responsible for the early flowering phenotype. To corroborate this, the phyB[WT], phyB [S86A] and phyB[S86D] transgenes were introgressed in the *ft-10* mutant (Yoo *et al.*, 2005). As shown in Figures S2 and S3, the *ft-10* mutation completely suppressed early flowering of phyB overexpressors in both SDs and LDs, supporting the idea that higher levels of *FT* are the cause of accelerated flowering of these lines. Photoperiodic signals are relayed to *FT* transcription mainly by CO, a key activator of *FT* expression (Samach *et al.*, 2000). To test if CO is required for increased *FT* transcription and early flowering of phyB overexpressors, the phyB transgenes were introgressed in the *co-9* mutant (Balasubramanian *et al.*, 2006) and homozygous progenies were analyzed in SDs. The loss of CO resulted in very low *FT* mRNA levels in all lines and completely eliminated the nightly peak of *FT* expression (Figure 3e). We showed that this is not due to altered accumulation of the phyB proteins in the *co-9* background (Figure S1a). Moreover, the *co-9* mutation abolished early flowering of phyB-overexpressing lines (Figures 3f and S3). Collectively these data suggest that phyB overexpression positively regulates CO at the post-translational level around dusk and during the night, which in turn induces *FT* transcription resulting in accelerated flowering.

### Induction of *FT* in the night requires the Pfr conformer of phyB

Phosphorylation of phyB at Ser-86 accelerates the dark reversion of the photoreceptor, thus in the night phyB Pfr levels decrease faster in phyB[S86D] or slower in phyB [S86A] lines expressing the phospho-mimic or the non-phosphorylatable mutant versions of phyB, respectively (Medzihradszky *et al.*, 2013). The sharp contrast between phyB[S86D] and phyB[S86A] plants in terms of molecular and physiological flowering phenotypes suggested that the nightly peak of *FT* expression in phyB[S86A] (and also in phyB[WT]) plants is related to relatively high and persisting levels of phyB Pfr. To test this more directly, plants were grown and harvested in SDs as before, but half of the seedlings were irradiated with far-red light for 1 h just before lights-off in order to convert phyB receptors to the Pr form. As expected, the far-red pulse eliminated the *FT* peak during the night in phyB[WT] and phyB[S86A] plants (Figure 4a) verifying the requirement of high levels of phyB Pfr to evoke the response. The far-red light treatment significantly attenuated the peak of *FT* around dusk (ZT9) in all phyB overexpressor lines (Figure 4a), but not in Col plants (Figure S4a). Since *CO* mRNA accumulation was not altered by the far-red light treatment in any of the lines (Figure S4b,c), these results corroborate and extend the previous ones, demonstrating that residual Pfr form of phyB in the dark stimulates CO at the post-translational level leading to increased *FT* expression and early flowering in SDs. The role of phyB Pfr in this response was also investigated in lines overexpressing two other phyB mutant derivatives in the *phyB-9* background. The Tyr276-His substitution creates a constitutively active version of phyB (phyB[Y276H]), which is present in the Pfr form independently of the light conditions (Su and Lagarias, 2007). In contrast, the Cys357Thr substitution eliminates the chromophore binding site and creates a constitutively inactive derivative of the receptor (phyB[C357T]), which is present in the Pr form independently of the light conditions (Clack *et al.*, 2009). phyB[Y276H] and phyB[C357T] were expressed at levels comparable with phyB[WT], phyB [S86A] and phyB[S86D] (Figure S4). Analysis of *CO* and *FT* mRNA accumulation and flowering time of these lines revealed that phyB[Y276H] phenocopied phyB[S86A], whereas phyB[C357T] plants were indistinguishable from *phyB-9* seedlings (Figure 4b–d). Moreover, *FT* levels were unaffected by the far-red light treatment in phyB[Y276H]

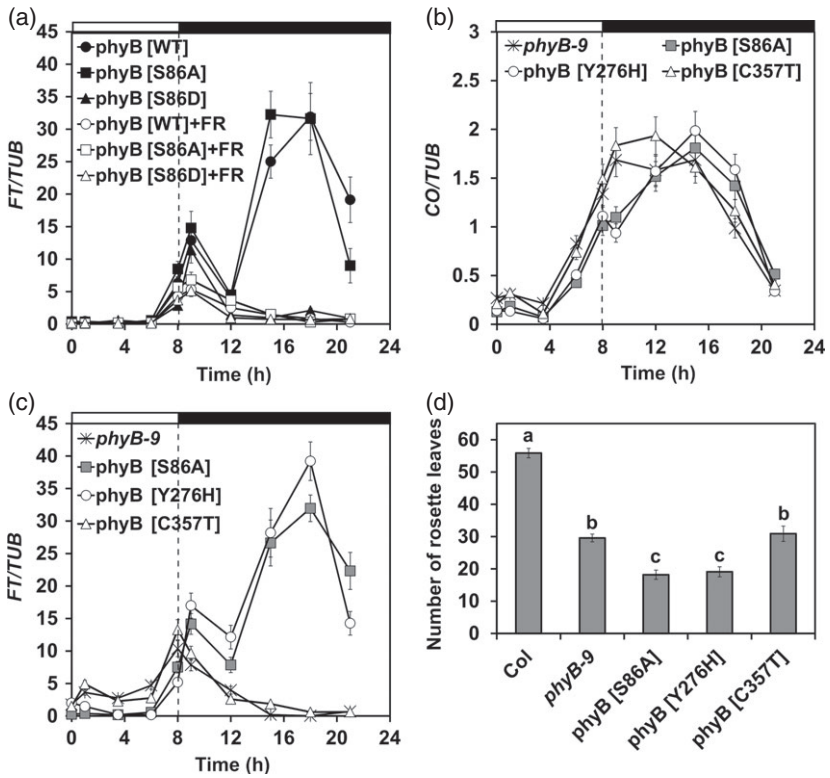


**Figure 4.** Induction of *FT* in the night requires phyB Pfr.

(a) The end-of-day far-red (EODFR) light treatment eliminates *FT* induction in the night. Plants were grown in 8 h light/16 h dark photocycles for 10 days. On day 10, half of the plants were treated with far-red light (30 μmol $m^{-2}$ $s^{-1}$) for 1 h before lights-off at ZT8 (+FR). *FT* and *TUB* mRNA levels were determined by qPCR assays. *FT* values normalized to the corresponding *TUB* values are plotted. White and black bars indicate light and dark conditions, respectively. Error bars represent standard error (SE).

(b, c) *CO* and *FT* mRNA levels under short-day (SD) conditions. Plants were grown under 8 h light/16 h dark photocycles for 10 days. *CO, FT* and *TUB* mRNA levels were determined by qPCR assays. *CO* (b) and *FT* (c) values normalized to the corresponding *TUB* values are plotted. White and black bars indicate light and dark conditions, respectively. Error bars represent SE.

(d) Flowering time under SD conditions. Plants were grown in 8 h light/16 h dark photocycles. Error bars indicate SE, and different letters show significant differences at $P < 0.01$ (Duncan's test).

plants (Figure S4a), demonstrating that *FT* induction at dusk and during the night is caused exclusively by phyB Pfr in the overexpressing lines. These results again indicate that a high level of phyB Pfr is required and sufficient for the observed flowering-related phenotypes of phyB overexpression.

### Up-regulation of CO function and *FT* transcription is likely mediated through the inhibition of COP1 activity by phyB Pfr

The results of the previous experiments suggested that the molecular basis of early flowering of phyB overexpressors is the reinforcement of CO function by phyB Pfr around dusk and in the night. CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) is an E3 ubiquitin ligase that promotes ubiquitination and subsequent degradation of CO protein at these times of the day (Jang *et al.*, 2008). Since COP1 activity is negatively regulated by photoactivated cryptochrome and phytochromes photoreceptors (Huang *et al.*, 2014; Lu *et al.*, 2015; Sheerin *et al.*, 2015), inhibition of COP1 by phyB Pfr could explain the observed flowering phenotypes. If this is the case, qualitatively similar molecular and physiological phenotypes are expected for *cop1* mutants and phyB[WT] or phyB[S86A] plants. To test this, *FT* mRNA levels and flowering time were determined in the *cop1-4* mutant in SDs. Abundance of *FT* mRNA was significantly, about 10-fold higher in *cop1-4* than in phyB [S86A] plants at any time point (Figure 5a). However, the pattern of *FT* accumulation in *cop1-4* was very similar to that in phyB[S86A]. Lower expression was detected during the day that increased rapidly after dusk and reached a peak around the middle of the night. According to the very high level of *FT* expression, *cop1-4* plants showed extreme early flowering, producing significantly less number of rosette leaves at bolting than phyB[S86A] plants (Figure 5b). These data are in agreement with previous findings (Jang *et al.*, 2008; Yu *et al.*, 2008) and support the hypothesis that phyB overexpression accelerates flowering by impairing COP1-mediated CO degradation.

COP1 functions in complex with SUPPRESSOR OF PHYA-105 1–4 (SPA1–4) proteins, where COP1 represents the catalytic subunit, whereas the primary role of SPA proteins is the modulation (enhancement) of COP1 activity via direct protein–protein interactions (Seo *et al.*, 2003; Ordonez-Herrera *et al.*, 2015). Recent studies demonstrated that binding of phyB Pfr to SPA1 disrupts COP1–SPA1 interaction resulting in lower ubiquitin ligase activity and accumulation of COP1 target proteins such as LONG HYPOCOTYL IN FAR-RED1 (HFR1) or ELONGATED HYPOCOTYL 5 (HY5) (Lu *et al.*, 2015; Sheerin *et al.*, 2015). We reasoned that if flowering phenotypes of the phyB-overexpressing lines were mediated by this mechanism, phyB[WT], phyB[S86A] and phyB[S86D] proteins should bind to SPA1 with different efficiencies during the early night, when phyB Pfr levels are getting limited. To test this, the different phyB derivatives and SPA1 were co-expressed and the interac-

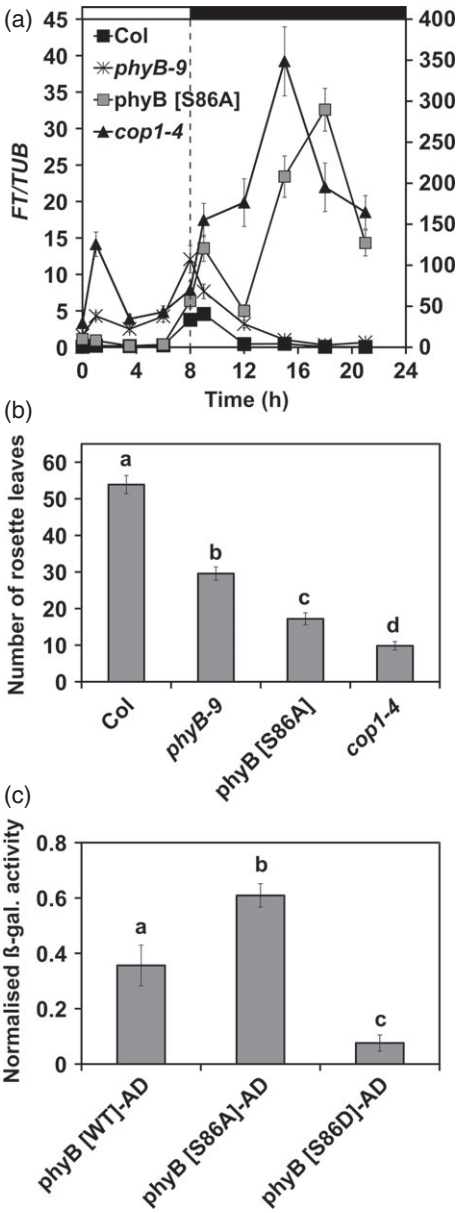


**Figure 5.** The *cop1-4* mutant displays molecular and physiological phenotypes similar to that of phyB[S86A].

(a) *FT* mRNA levels under short-day (SD) conditions. Plants were grown in 8 h light/16 h dark photocycles for 10 days. *FT* and *TUB* mRNA levels were determined by qPCR assays. *FT* values normalized to the corresponding *TUB* values are plotted. *FT* values from the *cop1-4* mutant are plotted on the secondary axis. White and black bars indicate light and dark conditions, respectively. Error bars represent standard error (SE).

(b) Flowering time under SD conditions. Plants were grown in 8 h light/16 h dark photocycles. Error bars indicate SE; $^{a-d}$different letters show significant differences at $P < 0.01$ (Duncan's test).

(c) phyB–SPA1 interaction in yeast. Yeast cells expressing BD–SPA1 and phyB[WT]–AD or phyB[S86A]–AD or phyB[S86D]–AD fusion proteins were grown on solid media supplemented with 20 μM PCB for 24 h. The cultures were either pulsed with red light (20 μmol $m^{-2}$ $s^{-1}$) for 10 min and returned to darkness for 6 h, or transferred to continuous red light (20 μmol $m^{-2}$ $s^{-1}$) for 6 h. Cells were harvested and β-galactosidase activities were determined. Values of the pulsed samples were normalized to those of the corresponding continuously irradiated samples. Error bars indicate SE; $^{a-c}$different letters show significant differences at $P < 0.01$ (Duncan's test).

tions analyzed in the yeast two-hybrid system (Figure 5c). In order to mimic conditions of the early night, yeast cells were cultured in darkness for 24 h, pulsed with red light for 10 min and returned to darkness for 6 h. Alternatively, yeast cultures were transferred to continuous red light for 6 h (saturating light conditions). β-Galactosidase activities of the pulsed samples were normalized to those measured under continuous irradiation to minimize the effects of differences in binding properties that are not related to dark reversion. Among the three phyB derivatives, phyB[S86A] showed the strongest retention of binding activity in darkness that was about two-fold or 10-fold higher than that of phyB[WT] or phyB[S86D], respectively (Figure 5c). Binding characteristics of the phyB variants correlated well with the molecular and physiological phenotypes of the lines expressing these proteins (Figures 1 and 3), suggesting that inhibition of COP1 activity by the phyB–SPA1 interaction is the primary mechanism underlying early flowering of phyB overexpressors.

## DISCUSSION

Besides promoting degradation of the CO protein, phyB has been shown to control flowering via a CO-independent pathway possibly involving PHYTOCHROME AND FLOWERING TIME 1 (PFT1) (Cerdan and Chory, 2003; Inigo *et al.*, 2012). As overexpression of phyB in the *co-9* or *ft-10* mutant backgrounds had no effect on flowering time (Figures 3 and S3), we concluded that this action of phyB is mediated exclusively by the CO/FT-dependent pathway. Consistent with this, we found elevated levels of *FT* mRNA in the phyB-overexpressing plants around dusk and during the night both in SDs and LDs (Figure 3). As *CO* mRNA levels were not affected, overexpressed phyB appeared to stabilize the CO protein at these times. In contrast, phyB facilitates degradation of CO in a red-light-dependent manner in the first half of the day in wild-type plants (Valverde *et al.*, 2004; Jang *et al.*, 2008). To see if the effect of overexpressed phyB is also Pfr-dependent, we first applied end-of-day far-red (EODFR) treatments that diminished accumulation of *FT* mRNA in the night and significantly reduced the peak of *FT* expression at dusk, verifying that up-regulation of *FT* at these times was due to overexpressed phyB Pfr (Figure 4). The EODFR treatment accelerates flowering in wild-type and several multiple *phy* mutant plants that led to the conclusion that Pfr forms of endogenous phyB, phyD and phyE, still present during the night, delay flowering (Bagnall *et al.*, 1995; Devlin *et al.*, 1998, 1999). This effect of EODFR was almost unchanged in the *co* mutant (Devlin *et al.*, 1998), but was reduced in *pft1* (Cerdan and Chory, 2003) reflecting the function of the CO-independent light-quality pathway downstream of phyB, PhyD and phyE (Inigo *et al.*, 2012). The single EODFR treatment caused only a moderate increase of *FT* mRNA levels in Col wild-type plants (Figure S3) that probably could not account for

early flowering. It is possible that either repeated and long-term use of EODFR or a more advanced developmental stage is required for the up-regulation of *FT* by this treatment or, as suggested by the early flowering of the *phyB phyD phyE ft* quadruple mutants (Inigo *et al.*, 2012), *FT* may not be the only integrator of signals downstream of phyB, phyD and phyE. Nevertheless, the EODFR treatment delayed rather than accelerated flowering in a phyB-overexpressing line (Bagnall *et al.*, 1995), consistently with our EODFR results.

Second, we analysed the molecular and physiological phenotypes of transgenic lines overexpressing mutant versions of phyB with conditionally or constitutively altered Pfr levels. Phosphorylation of phyB at Ser-86 accelerates dark reversion of the receptor that inhibits signaling under non-saturating light conditions by lowering Pfr levels (Medzihradszky *et al.*, 2013). We showed that overexpression of the phospho-mimic version of phyB (phyB[S86D]) induced *FT* expression at dusk, but not during the night. Physiological and molecular data (Figures 1 and 3) suggested that induction of *FT* around dusk or in the night is the main cause of accelerated flowering in LDs or SDs, respectively. Consistently, flowering of phyB[S86D] plants, relative to phyB[WT] or phyB[S86A] plants, was dramatically delayed in SDs, but not in LDs. The phyB[Y276H] or phyB[C357T] plants expressing constitutively Pfr or Pr forms of phyB, respectively, phenocopied phyB[S86A] or *phyB-9* plants in terms of both *FT* expression and flowering time. These results clearly demonstrate that flowering phenotypes of phyB overexpressors depend on the active form of the receptor. Moreover, these results indicate that the decline of *FT* mRNA in phyB[WT], phyB[S86A] plants in the second half of the night of SD is not due to decreasing amounts of Pfr forms.

Our data strongly suggested that high levels of phyB Pfr stabilize the CO protein around dusk and during the night. The COP1–SPA ubiquitin ligase complex regulates CO protein levels at these times (Laubinger *et al.*, 2006; Jang *et al.*, 2008). The four SPA proteins (SPA1–SPA4) redundantly enhance the ubiquitin ligase activity of COP1 via physical interactions (Huang *et al.*, 2014). SPA1 and SPA4 were shown to be the primary SPA proteins controlling flowering time (Ordonez-Herrera *et al.*, 2015). The *cop1* and *spa* mutants flower early especially in SDs and have increased levels of CO protein and *FT* mRNA at dusk, but particularly during the night (Laubinger *et al.*, 2006; Jang *et al.*, 2008; Ordonez-Herrera *et al.*, 2015) (Figure 5). These phenotypes are qualitatively very similar to those observed for the phyB-overexpressing lines. Therefore, we proposed that phyB Pfr accelerates flowering by partial inhibition of the function of the COP1–SPA complex. Different photoreceptors were shown to reduce the activity of this ubiquitin ligase complex via direct interactions. CRY1 and CRY2 interact with SPA1, inhibiting COP1 function albeit by different mechanisms (Lian *et al.*, 2011; Zuo *et al.*, 2011). More recently, it has been demonstrated that the Pfr conformer of phyA and phyB binds to SPA1, disrupting the SPA1–COP1 interaction, resulting in lower activity of COP1 and accumulation of target proteins like HFR1 or HY5 (Lu *et al.*, 2015; Sheerin *et al.*, 2015). Using yeast two-hybrid assays and light conditions where Pfr levels are limited, we showed that the binding efficiency of SPA1 to the wild-type or phospho-mutant derivatives of phyB is tightly correlated with *FT* mRNA levels measured during the night in the transgenic lines overexpressing the corresponding phyB derivatives. This finding strongly suggests that overexpressed phyB Pfr controls CO protein levels and flowering time by impairing the SPA1–COP1 interaction.

In contrast to the effect of overexpression, phyB Pfr in wild-type plants promotes degradation of CO during the day. Since CO is ubiquitinated and degraded by the proteasome (Valverde *et al.*, 2004; Lazaro *et al.*, 2012), phyB is expected to positively modulate the function of an ubiquitin ligase other than COP1. The HOS1 Ring-finger type E3 ubiquitin ligase and the F-box protein ZTL, as a component of the $SCF^{ZTL}$ E3 ubiquitin ligase complex, regulate the turnover of CO in the morning and the first half of the day (Lazaro *et al.*, 2012; Song *et al.*, 2014). Neither genetic/functional interactions between phyB and HOS1 nor light regulation of HOS1 activity have been reported so far. Although ZTL interacts with phyB (Jarillo *et al.*, 2001; Kevei *et al.*, 2006), which could mediate the effect of the receptor on CO stability, the requirement of ZTL for this action of phyB has not been demonstrated yet. Nevertheless, due to the lack of this control, *FT* levels were elevated in the *phyB-9* mutant during the day (Figure 3). This phenotype was fully complemented by all phyB derivatives, demonstrating that the relatively weak early flowering phenotype of phyB [S86D] plants did not arise from partial complementation of the *phyB-9* mutant, and that overexpression of phyB at this time of the day could not induce *FT* expression.

These data collectively suggest that the net effect of phyB on CO turnover is determined by the time of the day, the level of phyB Pfr and the particular ubiquitin ligase controlled by phyB Pfr. In the first half of the day phyB promotes degradation of CO by enhancing the function of the unidentified ubiquitin ligase. This overrides the effect of inhibition of the COP1–SPA complex resulting in low CO levels. In the second half of the day and around dusk the function of the unidentified ubiquitin ligase is less dominant, which may be due at least in part to the action of PHL antagonizing the effect of phyB. However, the elevated *FT* levels in the *phyB-9* mutant indicate that this function is not totally absent yet. Overexpression of phyB induces rather than reduces *FT* levels, indicating the increasing effect of the inhibition of COP1–SPA at this time. In the night, the COP1–SPA complex has the prevailing effect on CO stability, thus phyB overexpression results in massive

*FT* induction. Our conclusions are summarized in Figure S5.

In contrast with its role in flowering time determination, the effect of phyB on the pace of the clock in continuous red light is proportional to the amount of the protein: phyB mutants show long period phenotypes, whereas phyB overexpressors display shorter periods (Hall *et al.*, 2002; Palagyi *et al.*, 2010). According to the estimated levels of Pfr forms, phyB[S86A] and phyB[S86D] plants produced shorter and longer periods as compared with phyB[WT] plants at lower fluences of red light, but periods were identical in these lines under saturating illumination. Interestingly, periods in phyB[S86D] plants matched the periods in *phyB-9* plants at fluence rates lower than 35 μmol $m^{-2}$ $s^{-1}$. In contrast, relative hypocotyl length in these two plants became identical at more than one order of magnitude lower fluences of red light (Medzihradszky *et al.*, 2013). These observations suggest that pace of the clock is much less sensitive to phyB Pfr than the control of hypocotyl elongation. We also observed that phyB[S86D] or phyB[S86A] plants produced fluence rate response curves with increased or reduced slopes, respectively, compared with phyB[WT] plants. It is tempting to speculate that fluence rate dependent phosphorylation of phyB at Ser-86 could contribute to parametric entrainment by red light.

## EXPERIMENTAL PROCEDURES

### Plant materials, growth conditions and light treatments

All plants were of the Columbia (Col) accession of *Arabidopsis thaliana.* The *phyB-9, co-9* and *ft-10* have been described (Reed and Chory, 1994; Yoo *et al.*, 2005; Balasubramanian *et al.*, 2006). Transgenic lines overexpressing the wild-type or the Ser86Ala and Ser86Asp mutant versions of phyB have been described (Medzihradszky *et al.*, 2013). Col and *phyB-9* lines expressing the *CCA1:LUC* or *GI:LUC* reporter genes have been described (Palagyi *et al.*, 2010). The *phyB-9* lines have been crossed with the phyB-overexpressing lines in order to have the same copy of the marker gene/insertion. The mutations for Tyr276His and Cys357Thr substitutions were introduced by using the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent, http://www.genomics.agilent.com/en/product.jsp?cid=AG-PT-175&tabId=AG-PR-1162&_requestid=90796) according to the manufacturer's instructions. The mutant genes were cloned between the *35S* promoter of the cauliflower mosaic virus and the *YFP* gene in the modified pPCV812 binary vector (Palagyi *et al.*, 2010). The constructs have been transformed in *phyB-9* plants (Clough and Bent, 1998). Homozygous T3 progenies with expression levels comparable to those of the phyB-overexpressing lines were selected for further experiments. For RNA or protein isolation and for luminescence assays surface sterilized seeds were sown on solidified Murashige and Skoog media supplemented with 3% sucrose. Seeds were incubated at 4°C for 3 days in darkness then transferred to 8 h light/16 h dark, 12 h light/12 h dark or 16 h light/8 h dark photocycles at 22°C (MLR-350H, Sanyo, Gallenkamp, UK). White light was provided by fluorescence tubes at 70–100 μmol $m^{-2}$ $s^{-1}$ fluence rate. Far-red ($\lambda_{max}$ = 735 nm) and blue light ($\lambda_{max}$ = 470 nm) were provided by Snap-Lite LED light sources (Quantum Devices, WI, USA). Far-red light was filtered through an RG 9 glass filter (Schott, Germany).

### Analysis of gene expression

Plants were grown for 10 days in the indicated photocycles before harvesting. Total RNA was isolated with the RNeasy Plant Mini Kit (Qiagen, https://www.qiagen.com/hu/shop/sample-technologies/rna-sample-technologies/total-rna/rneasy-plant-mini-kit); 1 μg RNA was used as template for reverse transcription done with the RevertAid RT Reverse Transcription Kit (Thermo Scientific, https://www.lifetechnologies.com/order/catalog/product/K1621). cDNA samples were diluted 1:5 and used as templates in quantitative real-time PCR assays employing Power SYBR Green Master Mix and an ABI Prism 7300 Real-Time PCR System (Life Technologies, http://www.lifetechnologies.com/hu/en/home/life-science/pcr/real-time-pcr/real-time-pcr-reagents/sybr-green-real-time-master-mixes/power-sybr-green-master-mix.html; https://products.appliedbiosystems.com/ab/en/US/adirect/ab?cmd=catNavigate2&catID=601250). All procedures were performed according to the manufacturer's instructions. Total protein extraction, western blot analysis and detection of YFP fusion proteins were done essentially as described previously (Medzihradszky *et al.*, 2013). The assays were repeated two or three times and representative data are shown.

### Luminescence and yeast two-hybrid assays

Luciferase activity was assessed by measuring single seedlings with an automated luminometer (TopCount NXT, Perkin Elmer, http://www.perkinelmer.com/catalog/family/id/topcount) for 2–7 days as described previously (Kevei *et al.*, 2006). For fluence rate curves, circadian periods of luminescence rhythms were measured in seedlings transferred to constant illumination of red light at the fluence rates indicated. All rhythm data were analyzed with the Biological Rhythms Analysis Software System (BRASS, available at http://millar.bio.ed.ac.uk/PEBrown/BRASS/BrassPage.htm). Variance-weighted mean periods within the circadian range (15–40 h) and SEMs were estimated as described. For phase shift experiments, 7-day-old plants were transferred to darkness for 33 h and treated with a 1 h red light pulse at 1 or 75 μmol $m^{-2}$ $s^{-1}$ fluence rate. Phase values were determined as the time of the second peak after the light pulse, and were normalized to free-running period length and are shown as circadian time (CT) (Salome *et al.*, 2002). Experiments were repeated three or four times. Yeast two-hybrid assays were performed as previously described (Sheerin *et al.*, 2015).

### Measurement of flowering time

Seeds were sown on soil and grown in SD (8 h light/16 h dark) or LD (16 h white light/8 h dark) conditions at 22°C. Flowering time was recorded as the number of rosette leaves at the time when inflorescences reached 1 cm height. Experiment was repeated twice or three times using 30–40 plants per genotype. For all data collected in this work, statistical significance was assessed by Duncan's Multiple Range Test calculated with the SIGMASTAT 3.5 software.

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## SUPPORTING INFORMATION

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

**Figure S1.** Levels of phyB fusion proteins in the transgenic lines used in this study.

**Figure S2.** The early flowering phenotype depends on FT.

**Figure S3.** CO and FT are epistatic to PHYB in long-day conditions.

**Figure S4.** The EODFR treatment does not affect transcription of *CO*.

**Figure S5.** Model for the role of phyB in the regulation of CO stability in wild-type and phyB-overexpressing plants.

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