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COP1 and ELF3 control circadian function and photoperiodic flowering by regulating GI stability

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SUMMARY

Seasonal changes in day length are perceived by plant photoreceptors and transmitted to the circadian clock to modulate developmental responses, such as flowering time. Blue light-sensing cryptochromes, the E3 ubiquitin-ligase COP1, and clock-associated proteins ELF3 and GI, regulate this process, although the regulatory link between them is unclear. Here, we present data showing that *COP1* acts with *ELF3* to mediate day length signaling from *CRY2* to *GI* within the photoperiod flowering pathway. We found that COP1 and ELF3 interact in vivo and show that ELF3 allows COP1 to interact with GI in vivo, leading to GI degradation in planta. Accordingly, mutation of *COP1* or *ELF3* disturbs the pattern of GI cyclic accumulation. We propose a model in which ELF3 acts as a substrate adaptor, enabling COP1 to modulate light input signal to the circadian clock through targeted destabilization of GI.

INTRODUCTION

Resetting (or synchronization) of the circadian clock to day-night cycles is essential in most flowering plants, since floral transition is mainly triggered by seasonal changes in photoperiod (Yanovsky and Kay, 2003). Based on their responsiveness to day lengths in the promotion of flowering, plants can be classified as long-day (LD), short-day (SD) and day-neutral plants.

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Arabidopsis is a facultative LD plant that flowers much earlier and with fewer leaves in LD (16 hr light/8 hr dark; 16L/8D) than in SD (8L/16D) (Simpson et al., 1999). Clock resetting by day length is mediated by members of the ZEITLUPE (ZTL), FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1) and LOV, KELCH PROTEIN 2 (LKP2) protein family (Nelson et al., 2000; Schultz et al., 2001; Somers et al., 2000). ZTL and FKF1 act as blue light receptors that interact with GIGANTEA (GI), a clock-associated protein whose accumulation is tightly controlled at both transcriptional and post-translational levels (Fowler et al., 1999; Kim et al., 2007; David et al., 2006; Sawa et al., 2007). Indeed, blue light stabilizes the FKF-GI and ZTL-GI interactions, allowing these complexes to regulate flowering time and circadian rhythms by controlling protein stability of a floral repressor CYCLING DOF FACTOR 1 (CDF1) and a circadian oscillator component TIMING OF CAB EXPRESSION 1 (TOC1), respectively (Imaizumi et al., 2005; Kim et al., 2007; Más et al., 2003; Sawa et al., 2007). Two additional classes of photoreceptors, the red/far-red light sensing phytochromes (Phy) and the blue/UVA-light sensing cryptochromes (CRY), enable clock resetting to day-night cycles in *Arabidopsis* (Somers et al., 1998). However, their regulatory mechanisms in controlling light input to the clock are not well understood.

The clock itself regulates light resetting of the oscillator by limiting the timing of maximum responsiveness to light to specific day periods, a phenomenon commonly referred to as gating (Millar and Kay, 1996). *EARLY FLOWERING 3* (*ELF3*) is a clock-associated gene that plays a pivotal role in the circadian gating pathway (Hicks et al., 1996; McWatters et al., 2000). Thus, *ELF3* rhythmically inhibits the activity of the light input pathways around dusk by reducing clock sensitivity to light resetting at this phase. However, the molecular mechanism on how *ELF3* product modulates this process remains largely unknown. *ELF3* encodes a nuclear protein of unknown biochemical activity which has been proposed to act as a transcriptional regulator to control the period length of expression of clock-controlled genes, including flowering-time regulators (Liu et al., 2001). Thus, lesions in *ELF3* cause both early flowering, possibly by increased accumulation of transcripts of floral inducers such as *CONSTANS* (*CO*) and *GI*, and arrhythmic expression of a morning-specific clock regulated gene *CHLOROPHYLL A/B BINDING 2* (*CAB2*) and an oscillator component *LATE ELONGATED HYPOCOTYL* (*LHY*) (Hicks et al., 2001; Kim et al., 2005; Schaffer et al., 1998; Suárez-López et al., 2001).

CONSTITUTIVE PHOTOMORPHOGENIC 1 (*COP1*) is also related to the control of light input to the clock, participating in the modulation of circadian rhythms and flowering transition in *Arabidopsis*. Weak mutations in *COP1* have no effect on flowering time under LD but cause early flowering under SD, which is referred to as photoperiod-insensitive early flowering (McNellis et al., 1994). In addition, weak *cop1* mutants display period-shortening of clock-controlled gene expression (Millar et al., 1995). *COP1* was first identified in *Arabidopsis* as a repressor of seedling photomorphogenesis in darkness (Deng et al., 1991). Indeed, COP1 acts as a RING-type E3 ubiquitin-ligase that mediates ubiquitination and targeted degradation of positive regulators of light signal transduction in dark conditions (Yi and Deng, 2005). This process is most likely repressed by physical interaction of CRY with COP1 under light conditions (Wang et al., 2001; Yang et al., 2001). It has been recently shown that COP1 represses flowering by promoting degradation of CO through the proteasome during the night (Jang et al., 2008; Liu et al., 2008). However, control of CO stability neither explains COP1 function on clock synchronization nor fully demonstrates COP1 implication in flowering-time control.

Here, we report that COP1 and ELF3 function towards GI destabilization plays an important role in the regulation of light input signaling to the clock and the control of the expression modes of flowering-time genes. Our findings provide new insights as to how the temporal

information of seasonal changes in day lengths is transferred from photoreceptors to the circadian clock to allow clock resetting and the transition to flowering in higher plants.

RESULTS

COP1 Acts between *CRY2* and *GI* in the Floral Inductive Pathways

To genetically dissect the role of *COP1* as a flowering repressor, we generated double and triple mutants that combined the weak *cop1-4* mutation – note that *cop1* null mutations are lethal – with representative mutations affecting the four major flowering-time regulatory pathways: photoperiod, autonomous, gibberellin and vernalization pathways (Figure 1A; Table S1 in the Supplemental Data available online). Based on flowering-time phenotypes in LD and SD, we found that *COP1* is placed downstream of *CRY2* since the late-flowering phenotype of *cry2-1* mutation completely disappeared in a *cop1-4* background (Figure 1B). When *gi-1 cop1-4* double mutants were analyzed, we found they flowered as late as *gi-1* plants, indicating that *GI* acts downstream of *COP1*. Previous reports showed that *gi* is epistatic to *elf3*, and these two mutations are epistatic to *cry2* (Chou and Yang, 1999; Fowler et al., 1999; Koornneef et al., 1998; Mockler et al., 1999; Zagotta et al., 1996). Altogether, these data indicate that *COP1* acts close to *ELF3* and both act between *CRY2* and *GI*.

The fact that *elf3-8 cop1-4* plants did not show an additive phenotype but rather flowered at the same time as *elf3-8* mutants further suggests that *ELF3* and *COP1* act on the same pathway. Definitive conclusions on their relative position cannot be drawn, since *elf3-8* and *cop1-4* alleles correspond to null and weak mutations, respectively (Hicks et al., 2001; McNellis et al., 1994). In this situation, lack of ELF3 activity rather than partial loss of COP1 function, should determine the flowering phenotype of the double mutant, independently of their order in the genetic pathway.

By contrast, an intermediate or additive flowering-time phenotype was observed in each double mutant of *cop1-4* and other mutations in flowering-time regulatory genes (Table S1), suggesting that these regulators act independently of *COP1*. However, in agreement with *FT* and *SUPRESSOR OF CONSTANS 1* (*SOC1*) functions as floral pathway integrators (Moon et al., 2005;Samach et al., 2000;Yoo et al., 2005), both photoperiod-insensitivity and early-flowering defects of *cop1-4* disappeared completely when introduced in a *ft-1 soc1-1* background (Figures 1A and 1B). Taken together, these results indicate that *COP1* functions as a flowering repressor as part of the phototransduction pathway that transfers light input signaling from *CRY2* to circadian clock-associated *GI* (Figure 1C), in agreement with recent reports showing that COP1 acts between CRY and CO within the photoperiodic pathway (Jang et al., 2008;Liu et al., 2008).

A Circadian Defect Causes Early Flowering in *cop1* Mutants

Day length-insensitive flowering can be reverted in some cases by using photoperiodic conditions that match the internal circadian period of the corresponding mutants, as shown for a central oscillator mutant *toc1-1* (Yanovsky and Kay, 2002). To test whether a circadian defect underlies the photoperiod-insensitive early flowering of *cop1* mutants, we examined whether the flowering-time phenotype of *cop1-4* mutants and that of plants overexpressing a dominant negative mutant version of COP1 (DN-COP1; Seo et al., 2004) can be rescued when they are entrained in LD (Light:Darkness; L:D = 2:1) and SD (L:D = 1:2) under reduced diurnal cycles, such as T (environmental time period) = 21 hr (21T) and 18T. Indeed, the flowering-time defectiveness of *cop1-4* and DN-COP1 mutants was greatly rescued under SD of 21T (7L/14D) and 18T (6L/12D), flowering much later and with more rosette leaves than under LD of 21T (14L/7D) and 18T (12L/6D), respectively (Figures 2A and 2B). We also examined the effect of reduced diurnal cycles in the flowering time of plants that combined *cop1-4* with the

loss-of-function allele *elf3-8* (Hicks et al., 2001). Loss of *ELF3* function causes light-conditional circadian arrhythmia and aphotoperiodic flowering (McWatters et al., 2000; Zagotta et al., 1996). Accordingly, the photoperiod-insensitive early flowering of *elf3-8* and *elf3-8 cop1-4* mutants persisted under LD and SD of 21T and 18T, although they flowered slightly later than those entrained in LD and SD of 24T (Figures 2A and 2B). These results suggest that the photoperiod-insensitive early flowering of *cop1* mutants is largely caused by a circadian defect.

cop1 Mutation Alters the Expression Modes of Multiple Flowering-Time Genes

To determine whether altered expression of clock-controlled flowering-time genes underlies the early flowering phenotype of *cop1* mutants, we analyzed the phase and amplitude of *ELF3*, *GI*, *CO* and *FT* rhythmic accumulation in wild-type (WT) and *cop1-4* plants entrained in both LD and SD. *ELF3* expression was rhythmic in *cop1-4* mutants under LD and SD, but the waveforms and amplitude of the oscillations differed from those in WT plants (Figure 3A). Notably, the abundance of *ELF3* mRNA in *cop1-4* mutants decreased rapidly in SD (Figure 3A, right panel). A similar effect was observed in the case of *GI* expression in *cop1-4* mutants grown under LD and SD (Figure 3B). Especially in SD, the timing of *GI* expression in *cop1-4* mutants began 2 hr earlier than that in WT plants (Figure 3B, right panel), possibly related to a 4 hr earlier decrease in *ELF3* mRNA levels.

In LD, *cop1-4* mutants showed almost the same phase of *CO* and *FT* expression as did WT plants (Figures 3C and 3D), although the peak time and waveform of *CO* expression were altered and the abundance of *FT* mRNA increased throughout the day. In SD, however, the onset of *CO* expression in *cop1-4* mutants shifted 4 hr earlier (Figure 3C, right panel), leading to elevated *CO* expression during daytime. Previous studies have proposed that earlier expression of *CO* coinciding with the light phase promotes *FT* expression and thereby induces photoperiod-insensitive flowering (Suárez-López et al., 2001; Yanovsky and Kay, 2002). Accordingly, advanced phase of *CO* expression prompts high *FT* expression in SD-grown *cop1-4* (Figure 3D, right panel) and *elf3-8* mutants (Figure S1), whereas *FT* transcript is absent in SD-grown WT plants (Figure 3D, right panel).

FLC negatively regulates flowering by repressing *FT* and *SOC1* expression through direct binding to their promoters (Searle et al., 2006). As reported previously (Nakagawa and Komeda, 2004), expression of *FLC* and *SOC1* was reduced and increased, respectively, in *cop1-4* and *elf3-8* mutants compared to that of WT plants independently of photoperiod conditions (Figure 3F), indicating that COP1 and ELF3 regulate *FLC* and *SOC1* expression. We therefore conclude that mutation of *COP1* results in a circadian defect that successively alters the peak time, daily rhythm, period length and/or mRNA abundance of flowering-time regulators, such as *ELF3*, *GI*, *CO* and *FLC*, and finally causes an increase in the expression of floral pathway integrators, *FT* and *SOC1*, leading thus to the photoperiod-insensitive early flowering of *cop1* mutants (Figures 1 and 2).

COP1 Mediates Ubiquitination and Proteasomal Degradation of ELF3

To examine the possible physical interactions between COP1, ELF3 and other circadian-clock and flowering-time regulators we utilized yeast two-hybrid assays (Figure 4A). We found that full-length COP1 interacted with ELF3 but not with any other proteins tested. Using the different domains of COP1 (Holm and Deng, 1999), we found that both RING-finger and coiled-coil (CC) domains of COP1 were sufficient for interaction with ELF3 (Figures 4B and S2A), especially with its N-terminal region (aa 1–261) (Figure S2B). To confirm their in vivo interaction, we performed a pull-down assay using transgenic *Arabidopsis* plants constitutively expressing a Tandem Affinity Purification (TAP)-tagged COP1 (TAP-COP1; Rubio et al., 2005). Immunodetection of endogenous ELF3 in the pull-down samples from TAP-COP1

plants, but not from TAP-GFP expressing plants, verified the in vivo interaction of COP1 and ELF3 (Figure 4C). TAP-COP1 plants treated with proteasome inhibitor MG132 exhibited much higher levels of TAP-COP1 and ELF3 proteins, suggesting that both COP1 and ELF3 are actively degraded by the proteasome.

Since COP1 has E3 ubiquitin (Ub)-ligase activity (Yi and Deng, 2005), we tested whether COP1 mediates ubiquitination of ELF3. For this, we performed in vitro ubiquitination assays using maltose binding protein-tagged COP1 (MBP-COP1) and gluthatione-S-transferase tagged ELF3 (GST-ELF3) proteins. As a result, we found that MBP-COP1 ubiquitinates GST-ELF3 in the presence of E1 Ub-activating and E2 Ub-conjugase activities (Figure 4D). These results prompted us to test whether ELF3 accumulation depends on COP1. With this aim, we transiently expressed HA-tagged ELF3 (HA-ELF3) and Flag-tagged COP1 (Flag-COP1) fusions in *Nicotiana benthamiana* leaves. A clear accumulation of HA-ELF3 was detected in leaf protein extracts when it was expressed in the absence of Flag-COP1. However, when HA-ELF3 and Flag-COP1 were co-expressed, the HA-ELF3 levels dropped abruptly, but not those of HA-GFP (negative control) (Figure 4E). As expected, a partial recovery of HA-ELF3 levels was observed when the co-infiltrated leaves were treated with MG132. Together, these results indicate that ELF3 is a target of COP1-mediated ubiquitination and regulated proteolysis through the proteasome.

COP1 Controls Photoperiod-dependent Fluctuation of ELF3 Abundance

Since ELF3 is ubiquitinated by COP1 and degraded by the proteasome (Figure 4), we analyzed whether COP1 controls ELF3 accumulation in vivo. Time-course analysis showed that periodic accumulation of ELF3 in WT plants almost follows the rhythmic pattern of *ELF3* mRNA expression in LD and SD (Figures 3A and 5A-C), consistent with previous observations using 12L/12D grown plants (Hicks et al., 2001;Liu et al, 2001). In *cop1-4* mutants grown under LD, ELF3 also accumulated rhythmically but its abundance was higher throughout the 24 hr cycle compared to that in WT plants (Figures 5A and 5C), which likely reflects changes in *ELF3* mRNA levels observed in these mutants grown under LD compared to WT plants (Figure 3A, left panel). An increase in ELF3 abundance was also observed in *cop1-4* mutants grown under SD at almost all time points compared to the WT (Figures 5B and 5C). However, in these conditions, *ELF3* mRNA levels were similar in WT and *cop1-4* plants (Figure 3A, right panel), likely indicating that circadian accumulation of ELF3 protein under SD is controlled by COP1.

Flowering and Circadian Effects of *ELF3* Overexpression Depend on *COP1*

It is noticeable that COP1 mediates ELF3 ubiquitination and degradation whereas our mutant analysis showed that COP1 has an agonistic, rather than antagonistic, effect on ELF3 in the control of photoperiodic flowering in SD (Figures 1, 4D and 4E; Table S1). To further assess the functional similarity of COP1 and ELF3, and to examine whether this similarity also extends to other clock-related traits, we tested the effect of *cop1* mutation on phenotypes caused by *ELF3* overexpression (ELF3-OX), such as late-flowering and period-lengthening of circadian gene expression (Covington et al., 2001; Liu et al., 2001; Kim et al., 2005). We found that ELF3-OX effect on flowering time was almost negligible in a *cop1-4* mutant background and that the expression patterns of *CO* and *FT* in SD-grown ELF3-OX *cop1-4* plants were similar to those in *cop1-4* mutants (Figures 5D and S3). Next, we examined the pattern of rhythmic accumulation of *LHY* mRNA in ELF3-OX *cop1-4* plants compared to that in each single parent and WT plants under constant light (LL) conditions. To this end, we performed free-running experiments by entraining plants to 12L/12D cycles and then transferring them to LL (Figures 5E and 5F). *LHY* transcripts continued to accumulate rhythmically in all lines tested under LL conditions. However, the onset and peak expression of *LHY* advanced 4 hr in *cop1-4* and ELF3-OX plants compared to WT plants. Further, the period length of *LHY* expression in *cop1-4* and ELF3-OX plants decreased and increased, respectively, according to

the altered period of clock-controlled gene expression reported for these lines (Covington et al., 2001; Millar et al., 1995). In addition, *LHY* expression waves showed increased amplitude throughout the time range of measurement and displayed an additional peak in ELF3-OX plants compared to those in both *cop1-4* and WT plants. Noticeably, the waveform and period length of *LHY* expression in ELF3-OX *cop1-4* plants more closely resembled those in *cop1-4* mutants, particularly during the early phase of the time course. Thus, we conclude that full COP1 activity is required for ELF3 function in the modulation of circadian rhythm and control of photoperiodic flowering.

COP1 and ELF3 Physically Interact with GI

Requirement of COP1 activity for ELF3 function to regulate circadian rhythms and flowering time (Figures 5D–F) and their physical interaction (Figures 4A–C) depict a model in which ELF3 and COP1 are placed at the same level within the photoperiodic pathway (Figure 1C). This raises the possibility that COP1 and ELF3 cooperatively control the activity of a target protein placed downstream both of them. To test this hypothesis, we first looked for physical interactions between ELF3 or COP1 and its downstream protein, GI. Using yeast two-hybrid assays, we found that ELF3 (bait) strongly interacted with full-length GI (prey) (data not shown). Especially, ELF3 interacted with the N-terminal (aa 1–507) and C-terminal (801–1173) regions of GI (Figure 6A). Notably, their physical interaction did not involve either the middle part of GI (401–907) or the C-terminal region of ELF3 (440–695). Although our yeast two-hybrid results did not reveal a physical interaction between full-length COP1 and GI (Figure 4A), we found that the RING-finger (1–104) and CC (121–209) domains of COP1 strongly interacted with the N-terminal region of GI (Figure 6B). Next, we looked for in vivo interactions between these proteins using bimolecular fluorescence complementation (BiFC) assays. Transient expression of cYFP-COP1 and nYFP-ELF3 (positive control), nYFP-ELF3 and GI-cYFP, or cYFP-COP1 and nYFP-GI in onion epidermal cells revealed MG132- and dark-dependent interactions for all these protein combinations (Figure 6C). Indeed, YFP fluorescence was not detected for any combination in the absence of MG132 or upon light incubation of transformed onion cells. Furthermore, we found that COP1 interaction with GI in the nucleus totally depends on the presence of ELF3, because of no detection of YFP fluorescence at all under any conditions without co-bombardment of HA-ELF3. These results indicate that ELF3 is essential for in vivo interaction of COP1 and GI, possibly acting as a protein adaptor, which likely explains why *elf3* mutants exhibit photoperiod-insensitively early-flowering phenotype in the presence of functional COP1 and GI.

COP1 and ELF3 Modulate Cyclic Accumulation of GI through Targeted Destabilization

GI stability is severely compromised upon light to dark transition, which coincides with COP1 and ELF3 accumulation in the nucleus (David et al., 2006). Moreover, proteasomal degradation of GI at night under both LD and SD conditions plays an important role in maintaining proper GI accumulation and function (Kim et al., 2007; Sawa et al., 2007). Based on these facts, we tested whether COP1 and ELF3 control GI stability in vivo. We transiently expressed by agro-infiltration derivatives of GI, ELF3 and COP1 (GI-GFP, HA-ELF3 and Flag-COP1) alone or in combination in *N. benthamiana* leaves. Immunoblots showed that GI-GFP completely disappeared when either HA-ELF3 or Flag-COP1 was present (Figure 6D, lanes 3 and 4). However, HA-ELF3 or Flag-COP1 did not affect HA-GFP levels (negative control), indicating that these two proteins trigger specific degradation of GI in vivo. Additionally, we confirmed that Flag-COP1 causes HA-ELF3 degradation (Figure 6D, lane 5). Further demonstration of COP1 and ELF3 functions in the control of GI stability was obtained from cell-free degradation assays (Figure 6E). Incubation of radiolabeled GI (TNT GI) with protein extracts corresponding to WT plants grown under LD conditions and harvested at ZT22, when GI is actively degraded by the proteasome (David et al., 2006), resulted in rapid degradation of TNT GI. However, the rate of TNT GI destabilization was lower when using similarly prepared

protein extracts from *cop1-4* mutants. An even lower rate was observed when *elf3-8* and *elf3-8 cop1-4* mutant extracts were used. By contrast, no significant differences were found in the rate of degradation of proteasome subunit RPT5 in the same plant extracts, indicating specificity for the effect of *cop1-4* and *elf3-8* mutations on GI degradation (Figure S4).

Next, we test whether COP1 and ELF3 participate in shaping the GI accumulation pattern of SD-grown plants. For this, we obtained *elf3-8* and *cop1-4* plants that overexpress a functional GI-GFP fusion (Kim et al., 2007) and compared their relative GI-GFP levels to those of transgenic WT plants throughout the SD cycle. As a result, we found that both mutations dramatically altered the GI-GFP waveform (Figure 6F). Thus, whereas GI-GFP accumulation peaked at ZT8 in WT plants to abruptly drop later on the day cycle, as previously shown (David et al., 2006: Kim et al., 2007: Sawa et al., 2007), in *elf3-8* plants, GI-GFP levels peaked earlier (ZT4) and remained higher than in WT plants for the rest of the time period studied. This result is in agreement with early accumulation of *FT* transcripts during daytime in *elf3-8* plants under SD (Figure S1). In *cop1-4* mutants, the effect on GI-GFP accumulation was even more drastic, with GI-GFP showing no clear cycling but almost constant levels that slightly drop during the night. Differences in GI-GFP accumulation pattern, or in the rate of TNT GI degradation, between *cop1-47* and *elf3-8* mutants might be due to the fact that the *cop1-4* allele corresponds to a weak mutation, which yields a partially functional COP1 protein (McNellis et al., 1994), whereas *elf3-8* allele corresponds to a null mutation (Hicks et al., 2001). However, mutation of *COP1* and *ELF3* may have additional effects that indirectly alter the GI accumulation pattern (i.e. by altering the function of other proteins that control GI stability). These indirect effects may be independent for each of these genes.

Together, these results depict a scenario where both COP1 and ELF3 regulate GI stability to shape the GI accumulation pattern, which is essential for proper GI function in controlling circadian oscillation and photoperiodism.

DISCUSSION

The regulatory mechanism that limits light input to the circadian clock is not well understood. CRY and ELF3 play opposite regulatory roles in this process, although the molecular basis of their function is unknown. In this study, we describe a regulatory mechanism that links COP1, an E3 Ub-ligase negatively controlled by CRY through direct interaction (Yang et al., 2001; Wang et al., 2001), and ELF3 with the control of GI stability to regulate clock resetting and photoperiodic flowering. Several pieces of evidence support this mechanism: First, similarly to *ELF3*, *COP1* acts downstream of *CRY2*, but independently of *PhyA* and *PhyB*, to confer photoperiodic information to the clock for the promotion of flowering (Table S1; Liu et al., 2001). Second, as in the case of *elf3*, *cop1* mutation causes circadian dysfunction that consequently affects the rhythmic expression of photoperiod-responsive genes, such as *GI*, *CO*, and *FT* (Figure 3; Kim et al., 2005; Suárez-López et al., 2001). Noticeably, both *cop1* and *elf3* mutations also reduce the transcript levels of *FLC* (Figure 3F), a repressor of flowering in the autonomous pathway, recently identified as a clock regulator (Edwards et al., 2006). This result is in accordance with a previous report showing that *ELF3* represses flowering, in part, through a *CO*-independent mechanism (Kim et al., 2005). Third, rescue of photoperiod-insensitive flowering in *cop1* mutants grown under reduced photoperiodic conditions depends on ELF3 function (Figure 2). Fourth, COP1 interacts with ELF3 in vivo (Figure 4C and 6C), and furthermore, COP1 activity is required for ELF3 function in the control of flowering time and circadian gene expression (Figure 5). Lastly, both COP1 and ELF3 physically interact with the same downstream target, GI, and control its accumulation in vivo, helping thus to shape the circadian oscillation pattern of GI (Figure 6).

GI accumulation follows the circadian pattern of its gene expression, which shows a peak in the late afternoon. At night, GI becomes destabilized through a proteasome-mediated process (David et al., 2006), which coincides with high accumulation of COP1 and ELF3 in the nucleus (Liu et al., 2001; von Arnim and Deng, 1994). Since function of GI-containing complexes, ZTL-GI and FKF1-GI, in the control of clock oscillation and photoperiodic flowering largely depends on the pattern of GI accumulation (Kim et al., 2007; Sawa et al., 2007), a negative control of GI stability by full activities of COP1 and ELF3 represents a plausible direct mechanism by which these two proteins regulate light input signaling to the circadian clock to repress flowering under SD conditions (Figure 7A). Therefore, based on our results, we propose that COP1 and ELF3 coordinately regulate flowering time and circadian rhythms by modulating the biological activity of GI on light-input signaling to the circadian clock (Figure 7A). Thus, the temporal COP1-ELF3-GI interaction and subsequent rapid degradation of GI at night may be crucial to shape the circadian profile of GI accumulation. In this way, COP1 and ELF3 would play a regulatory role in determining the unfavorable photoperiods for flowering, by preventing the incorrect timing of *CO* expression. In this scenario, antagonistic action of CRY2 and COP1 on GI degradation must be essential for the regulation of circadian expression of flowering-time genes downstream of *GI*. Further studies should shed light on the molecular mechanism that underlies CRY control of COP1 function towards ELF3 and GI.

By allowing COP1 and GI interaction, ELF3 likely acts as a substrate adaptor for COP1 action on GI in a concentration-dependent manner. Accordingly to this idea, ELF3 overexpression causes late flowering in a WT background (Figure 5D), possibly by promoting constitutive degradation of GI in the presence of physiological levels of COP1 (Figure 6D, lane 3). It has been shown that proteins modulating the activity of E3 Ub-ligases, such as substrate adaptors, can be also ubiquitinated and degraded by the proteasome (for a review, see Wu et al., 2006). Thus, ELF3-mediated interaction of COP1 with GI may result in degradation of not only the protein target GI, but also of the substrate adaptor ELF3. Indeed, we found that COP1 ubiquitinates ELF3 in vitro and triggers its degradation in vivo (Figures 4D and 4E). This regulatory process might prevent the endless recycling of active ELF3 and thereby limit the temporal extent of ELF3 function, helping thus to ensure circadian activation of ELF3. In agreement with this idea, we found that COP1 modulates rhythmic accumulation of ELF3, at least under SD conditions (Figure 5).

Finally, it has been recently shown that COP1 ubiquitinates and triggers proteasomal degradation of floral inducer CO, especially during nighttime in SD (Jang et al., 2008; Liu et al., 2008). In this process, blue-light active CRY likely stabilize CO by inhibiting COP1, in agreement with previous results showing that CO accumulates under blue light conditions and is degraded in darkness (Valverde et al., 2004). CO accumulation at night is required for *FT* expression and flowering transition under LD inductive conditions. COP1 role in the control of CO stability may help to explain why the photoperiod-insensitive phenotype of *cop1* mutants was not fully reverted when grown under reduced photoperiod conditions that closely match their internal circadian period (Figure 2). Based on these facts, COP1 regulation of CO activity, to repress flowering, would imply control of *CO* expression at the transcriptional level, through an ELF3-mediated pathway involving GI inactivation, and secondly, at the post-translational level by directly controlling CO stability (Figure 7B).

EXPERIMENTAL PROCEDURES

Detailed methods are presented in the Supplemental Data available online.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Chou ML, Yang CH. Late-flowering genes interact with early-flowering genes to regulate flowering time in *Arabidopsis thaliana*. Plant Cell Physiol 1999;40:702–708. [PubMed: 10501030]

Covington MF, Panda S, Liu XL, Strayer CA, Wagner DR, Kay SA. ELF3 modulates resetting of the circadian clock in *Arabidopsis*. Plant Cell 2001;13:1305–41315. [PubMed: 11402162]

David KM, Armbruster U, Tama N, Putterill J. Arabidopsis GIGANTEA protein is post-transcriptionally regulated by light and dark. FEBS Lett 2006;580:11934–1197.

Deng XW, Caspar T, Quail PH. *cop1*: a regulatory locus involved in light-controlled development and gene expression in *Arabidopsis*. Genes Dev 1991;5:11724–1182.

Edwards KD, Anderson PE, Hall A, Salathia NS, Locke JC, Lynn JR, Straume M, Smith JQ, Millar AJ. *FLOWERING LOCUS C* mediates natural variation in the high-temperature response of the *Arabidopsis* circadian clock. Plant Cell 2006;18:639–650. [PubMed: 16473970]

Fowler S, Lee K, Onouchi H, Samach A, Richardson K, Morris B, Coupland G, Putterill J. *GIGANTEA*: a circadian clock-controlled gene that regulates photoperiodic flowering in *Arabidopsis* and encodes a protein with several possible membrane-spanning domains. EMBO J 1999;18:4679–4688. [PubMed: 10469647]

Hicks KA, Millar AJ, Carre IA, Somers DE, Straume M, Meeks-Wagner DR, Kay SA. Conditional circadian dysfunction of the *Arabidopsis early-flowering 3* mutant. Science 1996;274:790–792. [PubMed: 8864121]

Hicks KA, Albertson TM, Wagner DR. *EARLY FLOWERING 3* encodes a novel protein that regulates circadian clock function and flowering in *Arabidopsis*. Plant Cell 2001;13:1281–1292. [PubMed: 11402160]

Holm M, Deng XW. Structural organization and interactions of COP1, a light-regulated developmental switch. Plant Mol Biol 1999;41:151–158. [PubMed: 10579483]

Imaizumi T, Schultz TF, Harmon FG, Ho LA, Kay SA. FKF1 F-box protein mediates cyclic degradation of a repressor of CONSTANS in Arabidopsis. Science 2005;309:293–297. [PubMed: 16002617]

Jang S, Marchal V, Panigrahi KC, Wenkel S, Soppe W, Deng XW, Valverde F, Coupland G. Arabidopsis COP1 shapes temporal pattern of CO accumulation conferring a photoperiodic flowering response. EMBO J 2008;27:1277–1288. [PubMed: 18388858]

Kim WY, Hicks KA, Somers DE. Independent roles for *EARLY FLOWERING 3* and *ZEITLUPE* in the control of circadian timing, hypocotyl length, and flowering time. Plant Physiol 2005;139:1557–1569. [PubMed: 16258016]

Kim WY, Fujiwara S, Suh SS, Kim J, Kim Y, Han L, David K, Putterill J, Nam HG, Somers DE. ZEITLUPE is a circadian photoreceptor stabilized by GIGANTEA in blue light. Nature 2007;449:356–360. [PubMed: 17704763]

Komeda Y. Genetic regulation of time to flower in *Arabidopsis thaliana*. Ann Rev Plant Biol 2004;55:521–535. [PubMed: 15377230]

Koornneef M, Alonso-Blanco C, Blankestijn-de Vries H, Peeters AJ. Genetic interactions among late-flowering mutants of *Arabidopsis*. Genetics 1998;148:885–892. [PubMed: 9504934]

Liu XL, Covington MF, Fankhauser C, Chory J, Wagner DR. *ELF3* encodes a circadian clock-regulated nuclear protein that functions in an *Arabidopsis PHYB* signal transduction pathway. Plant Cell 2001;13:1293–1304. [PubMed: 11402161]

Liu LJ, Zhang YC, Li QH, Sang Y, Mao J, Lian HL, Wang L, Yang QH. COP1-mediated ubiquitination of CONSTANS is implicated in cryptochrome regulation of flowering in *Arabidopsis*. Plant Cell 2008;20:292–306. [PubMed: 18296627]

Más P, Kim WY, Somers DE, Kay SA. Targeted degradation of TOC1 by ZTL modulates circadian function in *Arabidopsis thaliana*. Nature 2003;426:567–570. [PubMed: 14654842]

McNellis TW, von Arnim AG, Araki T, Komeda Y, Misera S, Deng XW. Genetic and molecular analysis of an allelic series of *cop1* mutants suggests functional roles for the multiple protein domains. Plant Cell 1994;6:487–500. [PubMed: 8205001]

McWatters HG, Bastow RM, Hall A, Millar AJ. The *ELF3 zeitnehmer* regulates light signalling to the circadian clock. Nature 2000;408:716–720. [PubMed: 11130072]

Millar AJ, Straume M, Chory J, Chua NH, Kay SA. The regulation of circadian period by phototransduction pathways in *Arabidopsis*. Science 1995;267:1163–1166. [PubMed: 7855596]

Millar AJ, Kay SA. Integration of circadian and phototransduction pathways in the network controlling *CAB* gene transcription in *Arabidopsis*. Proc Natl Acad Sci USA 1996;93:15491–15496. [PubMed: 8986839]

Mockler TC, Guo H, Yang H, Duong H, Lin H. Antagonistic actions of *Arabidopsis* cryptochromes and phytochrome B in the regulation of floral induction. Development 1999;126:2073–2082. [PubMed: 10207133]

Moon J, Lee H, Kim M, Lee I. Analysis of flowering pathway integrators in *Arabidopsis*. Plant Cell Physiol 2005;46:292–299. [PubMed: 15695467]

Nakagawa M, Komeda Y. Flowering of *Arabidopsis cop1*mutants in darkness. Plant Cell Physiol 2004;45:398–406. [PubMed: 15111714]

Nelson DC, Lasswell J, Rogg LE, Cohen MA, Bartel B. FKF1, a clock-controlled gene that regulates the transition to flowering in Arabidopsis. Cell 2000;101:331–340. [PubMed: 10847687]

Rubio V, Shen Y, Saijo Y, Liu Y, Gusmaroli G, Dinesh-Kumar SP, Deng XW. An alternative tandem affinity purification strategy applied to *Arabidopsis*protein complex isolation. Plant J 2005;41:767–778. [PubMed: 15703063]

Saijo Y, Sullivan JA, Wang H, Yang J, Shen Y, Rubio V, Hoecker U, Deng XW. The COP1-SPA1 interaction defines a critical step in phytochrome A-mediated regulation of HY5 activity. Genes Dev 2003;17:2642–2647. [PubMed: 14597662]

Samach A, Onouchi H, Gold SE, Ditta GS, Schwarz-Sommer Z, Yanovsky MF, Coupland G. Distinct roles of CONSTANS target genes in reproductive development of *Arabidopsis*. Science 2000;288:1600–1602. [PubMed: 10858139]

Sawa M, Nusinow DA, Kay SA, Imaizumi T. FKF1 and GIGANTEA complex formation is required for day-length measurement in Arabidopsis. Science 2007;318:261–265. [PubMed: 17872410]

Schaffer R, Ramsay N, Samach A, Corden S, Putterill J, Carre IA, Coupland G. The *late elongated hypocotyl* mutation of *Arabidopsis* disrupts circadian rhythms and the photoperiodic control of flowering. Cell 1998;93:1219–1229. [PubMed: 9657154]

Schultz TF, Kiyosue T, Yanovsky M, Wada M, Kay SA. A role for LKP2 in the circadian clock of Arabidopsis. Plant Cell 2001;13:2659–2670. [PubMed: 11752379]

Searle I, He Y, Turck F, Vincent C, Fornara F, Krober S, Amasino RA, Coupland G. The transcription factor FLC confers a flowering response to vernalization by repressing meristem competence and systemic signaling in *Arabidopsis*. Genes Dev 2006;20:898–912. [PubMed: 16600915]

Seo HS, Watanabe E, Tokutomi S, Nagatani A, Chua NH. Photoreceptor ubiquitination by COP1 E3 ligase desensitizes phytochrome A signaling. Genes Dev 2004;18:617–622. [PubMed: 15031264]

Simpson GG, Gendall AR, Dean C. When to switch to flowering. Annu Rev Cell Dev Biol 1999;15:519–550. [PubMed: 10611971]

Somers DE, Devlin PF, Kay SA. Phytochromes and cryptochromes in the entrainment of the *Arabidopsis* circadian clock. Science 1998;282:1488–1490. [PubMed: 9822379]

Somers DE, Schultz TF, Milnamow M, Kay SA. ZEITLUPE encodes a novel clock-associated PAS protein from Arabidopsis. Cell 2000;101:319–329. [PubMed: 10847686]

Suárez-López P, Wheatley K, Robson F, Onouchi H, Valverde F, Coupland G. *CONSTANS* mediates between the circadian clock and the control of flowering in *Arabidopsis*. Nature 2001;410:1116–1120. [PubMed: 11323677]

Valverde F, Mouradov A, Soppe W, Ravenscroft D, Samach A, Coupland G. Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. Science 2004;303:1003–1006. [PubMed: 14963328]

von Arnim AG, Deng XW. Light inactivation of Arabidopsis photomorphogenic repressor COP1 involves a cell-specific regulation of its nucleocytoplasmic partitioning. Cell 1994;79:1035–1045. [PubMed: 8001131]

Wang H, Ma LG, Li JM, Zhao HY, Deng XW. Direct interaction of *Arabidopsis* cryptochromes with COP1 in light control development. Science 2001;294:154–158. [PubMed: 11509693]

Wu JT, Chan YR, Chien CT. Protection of cullin-RING E3 ligases by CSN-UBP12. Trends Cell Biol 2006;16:362–369. [PubMed: 16762551]

Yang HQ, Tang RH, Cashmore AR. The signaling mechanism of *Arabidopsis* CRY1 involves direct interaction with COP1. Plant Cell 2001;13:2573–2587. [PubMed: 11752373]

Yanovsky MJ, Kay SA. Molecular basis of seasonal time measurement in *Arabidopsis*. Nature 2002;419:308–312. [PubMed: 12239570]

Yanovsky MJ, Kay SA. Living by the calendar: How plants know when to flower. Nat Rev Mol Cell Biol 2003;4:265–275. [PubMed: 12671649]

Yi C, Deng XW. COP1-from plant photomorphogenesis to mammalian tumorigenesis. Trends Cell Biol 2005;15:618–625. [PubMed: 16198569]

Yoo SK, Chung KS, Kim J, Lee JH, Hong SM, Yoo SY, Lee JS, Ahn JH. *CONSTANS*activates *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* through *FLOWERING LOCUS T* to promote flowering in *Arabidopsis*. Plant Physiol 2005;139:770–778. [PubMed: 16183837]

Zagotta MT, Hicks KA, Jacobs CI, Young JC, Hangarter RP, Meeks-Wagner DR. The *Arabidopsis ELF3* gene regulates vegetative photomorphogenesis and the photoperiodic induction of flowering. Plant J 1996;10:691–702. [PubMed: 8893545]

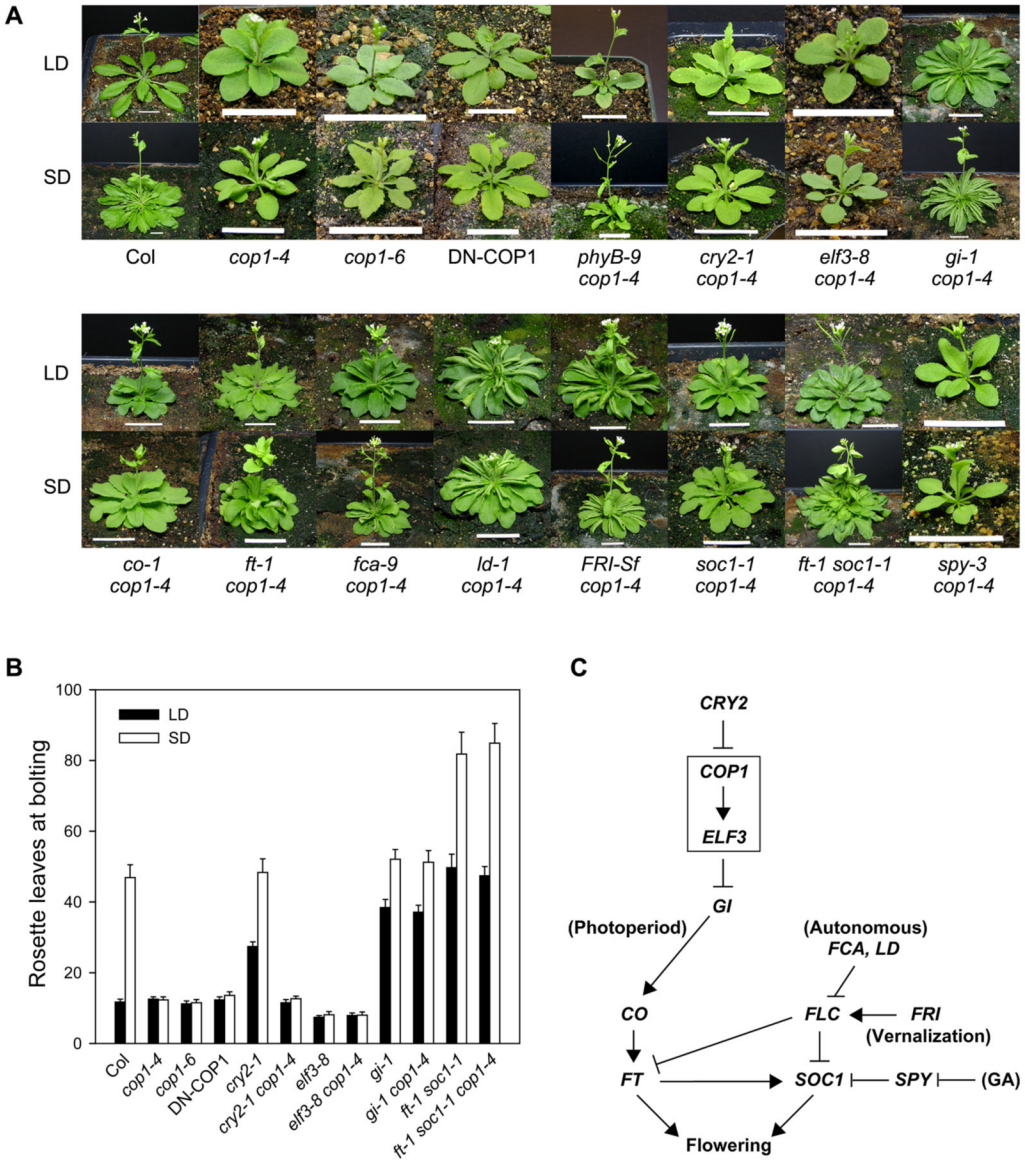

Figure 1. Genetic Analysis of *COP1* Function in the Control of Floral Induction

(A) Phenotypes of *cop1* mutants (*cop1-4*, *cop1-6*, and DN-COP1), and of double and triple mutants of *cop1-4* with different flowering-time mutants corresponding to the four genetic pathways of floral induction (see Table S1). Plants were grown at 22°C under cool-white fluorescent light (100 μmol m^{-2} s^{-1}) in LD (16L/8D) and SD (8L/16D), and photographed at 2 to 3 d after bolting. Bars = 2 cm.

(B) Epistatic relationship between *cop1-4* and *cry2-1*, *elf3-8*, *gi-1*, and *ft-1 soc1-1* mutations in the regulation of flowering time. Flowering time was measured as the number of rosette leaves at bolting (see Table S1).

(C) Genetic model of *COP1* regulation in floral induction pathways. *COP1* influences flowering time by mediating light input signaling from *CRY2* to *GI*. Based on our genetic data, epistatic relationships between *COP1* and *ELF3* cannot be definitively drawn. Thus, *COP1* and *ELF3* may act sequentially or at the same level (shown as a square enclosing both *COP1* and *ELF3*). Genetic interactions previously described in the literature are also shown (for a review, see Komeda, 2004).

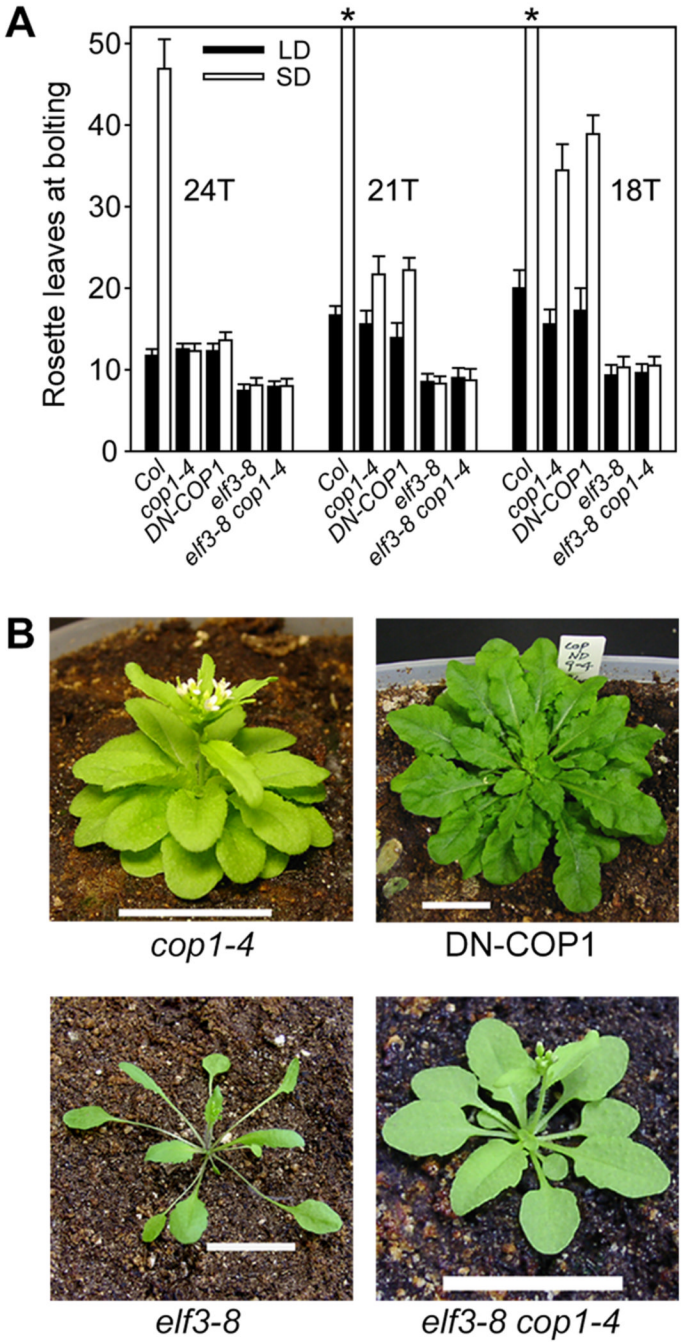

Figure 2. Flowering-time Defect of *cop1* Mutants Results from Circadian Dysfunction

(A) Effect of reduced photoperiods in the flowering time of *cop1-4*, DN-COP1, *elf3-8*, and *elf3-8 cop1-4* plants. Plants were entrained in LD (L:D = 2:1) and SD (L:D = 1:2) of 24 hr (24T; LD = 16L/8D, SD = 8L/16D), 21 hr (21T; LD = 14L/7D, SD = 7L/14D), and 18 hr (18T; LD = 12L/6D, SD = 6L/12D). Asterisks indicate that WT (Col) plants grown in SD (21T and 18T) had yet not bolt when more than 55 rosette leaves were counted. Mean and standard deviation values of at least 15 plants are shown. (B) Phenotypes of *cop1-4*, DN-COP1, *elf3-8*, and *elf3-8 cop1-4* mutants at bolting under SD of 18T. Plants were grown at 22°C under cool-white fluorescent light (100 μmol m^{-2} s^{-1}). Bars = 2 cm.

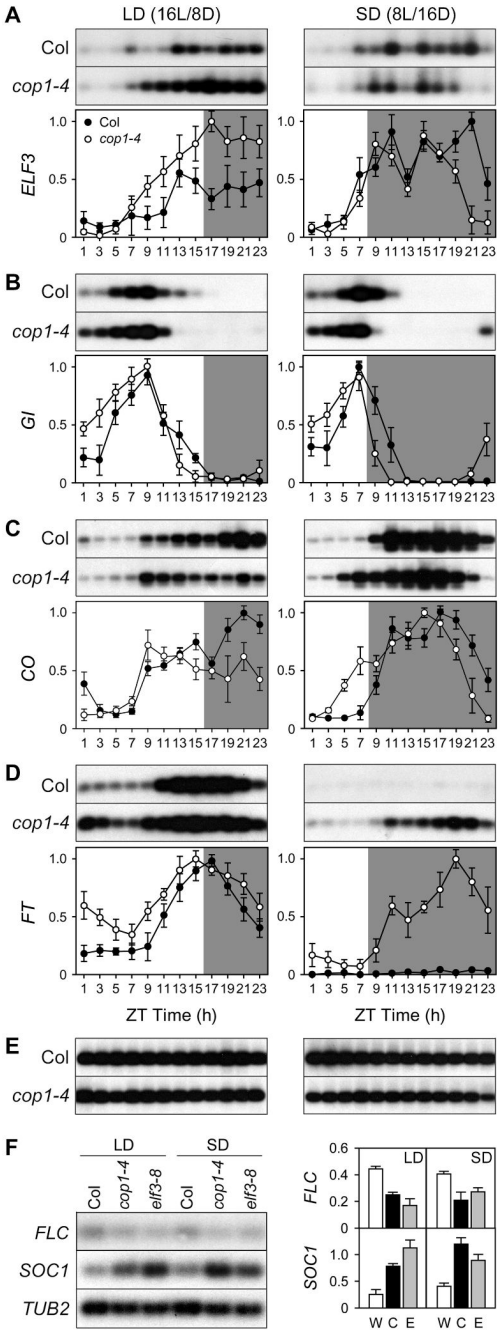

Figure 3. *cop1* Mutation Alters Gene Expression of Flowering-time Regulators

(A–D) Rhythmic patterns of *ELF3* (A), *GI* (B), *CO* (C) and *FT* (D) mRNA abundance in WT (Col) and *cop1-4* mutant plants. Total RNA samples were collected every 2 hr from 10-d-old and 20-d-old plants entrained in LD and SD of 24 hr, respectively. mRNA abundance was quantified by semiquantitative RT-PCR and expressed relative to the abundance of *TUBULIN2* (*TUB2*) transcripts (E). Grey areas behind the traces represent night periods. (F) Abundance of *FLC* and *SOC1* transcripts in WT (Col), *cop1-4* and *elf3-8* mutants. Equal amounts of total RNA corresponding to time points ranging from 1 to 23 hr were mixed for templates of semiquantitative RT-PCR. W, WT (Col); C, *cop1-4*; E, *elf3-8*.

(A–F) Plants were grown at 22°C under cool-white fluorescent light (100 μmol m^{-2} s^{-1}). Mean and standard deviation values of three replicates are shown.

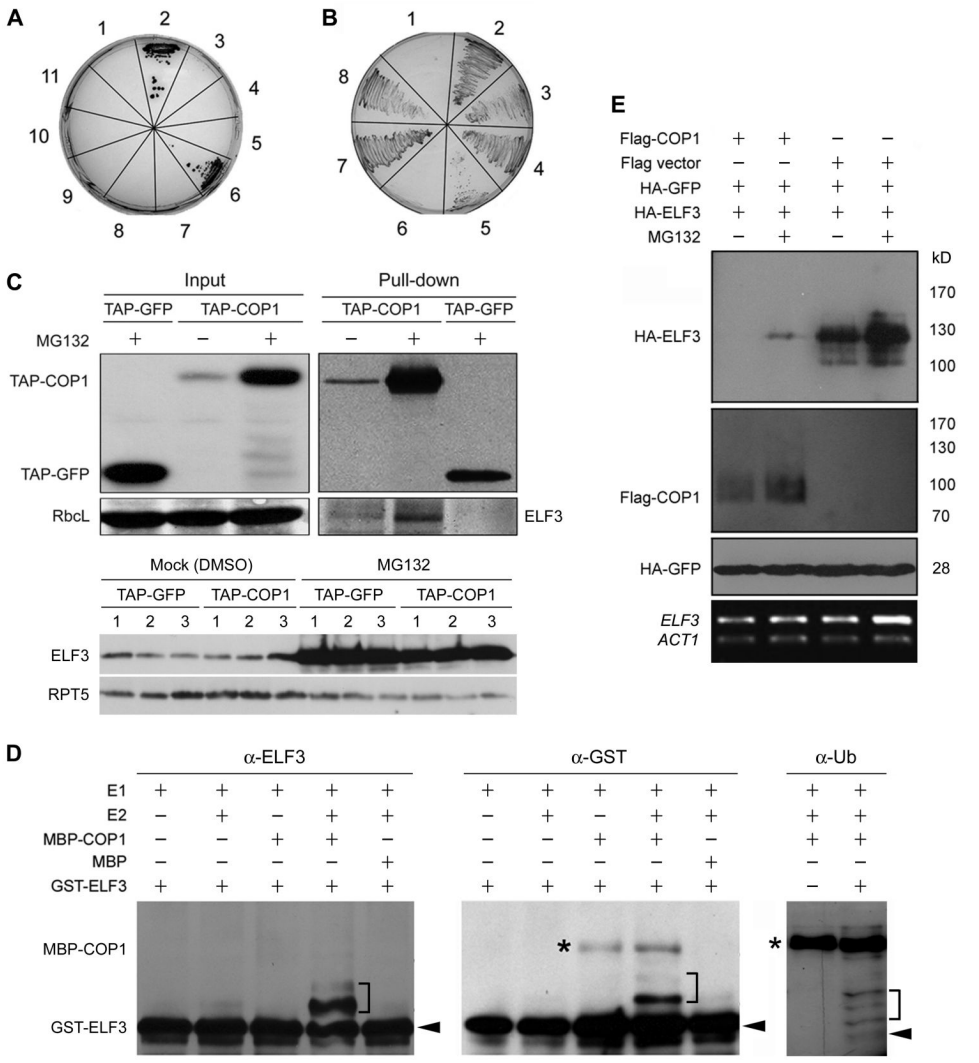

Figure 4. COP1 Interacts with and Ubiquitinates ELF3

(A) COP1 interacts with ELF3 in yeast two-hybrid assays. COP1 was used as bait in pGBK vector. The following preys, cloned into pGAD vector, were used: empty pGAD, as a negative control (1), CCT1, for positive control (2), LHY (3), CCA1 (4), TOC1 (5), ELF3 (6), ZTL (7), FKF1 (8), LKP2 (9), GI (10), and CO (11). Full-length cDNAs were used except for CCT1. CCT1 (A, B) represents the C-terminal domain of CRY1 (aa 486–681)

(B) ELF3 interacts most strongly with the RING-finger domain of COP1 in yeast two-hybrid assays. Baits in pGBK vectors and preys in pGAD vectors (bait::prey) were co-transformed into yeast such as full-length COP1::empty pGAD vector for negative control (1), p53::T for positive control (Clontech) (2), COP1::ELF3 (3), RING-finger domain of COP1 (aa 1–104)::ELF3 (4), coiled-coil domain of COP1 (121–213)::ELF3 (5), seven WD-40 repeat domain of COP1 (371–675)::ELF3 (6), COP1::CCT1 (7), and COP1::CCT2 (8). CCT2 represents the C-terminal domain of CRY2 (501–612). CCT1 and CCT2 were used as positive controls.

(C) In vivo interaction between COP1 and ELF3. Immunoblots of inmunoprecipitated samples from TAP-COP1 and TAP-GFP plants (upper panels). Anti-myc (to detect TAP-tagged proteins) and anti-ELF3 antibodies were used. RbcL protein levels were visualized as input control (total soluble protein) by Coomassie Brilliant Blue staining. A comparison of ELF3

levels (only detectable in nuclear extracts; Liu et al., 2001) in three different samples of TAP-COP1 and TAP-GFP plants (20 μg nuclear protein/lane) and the effect of proteasome inhibitor MG132 (50 μM) on ELF3 stability are also shown (bottom panels). Both ELF3 and RPT5 (loading control) were detected by ECL system.

(D) COP1 ubiquitinates ELF3 in vitro. GST-ELF3 ubiquitination assays were performed using MBP-COP1 (or MBP as a negative control), rice E2 Rad6 (E2), and yeast E1 (E1; Boston Biochem). Assay conditions were as previously described (Saijo et al., 2003). Ubiquitinated GST-ELF3 was detected using anti-ELF3 (left panel), anti-GST (middle panel) or anti-Ub (right panel) antibodies. Asterisks in middle and right panels indicate the position of MBP-COP1 (non-specific reaction of anti-GST) and ubiquitinated MBP-COP1 (anti-Ub), respectively.

(E) COP1 and the proteasome control ELF3 stability in vivo. Immunoblot analysis of protein extracts corresponding to agro-infiltrated *N. benthamiana* leaves with indicated plasmids in the presence or absence of MG132 (25 μM). HA-ELF3 (upper panel) and HA-GFP (input control; middle lower panel) were detected using anti-HA antibody, and Flag-COP1 (middle upper panel) using anti-Flag antibody. *HA-ELF3* (*ELF3*) and *ACTIN1* (*ACT1*) mRNA expression levels were analyzed by competitive RT-PCR (bottom panel).

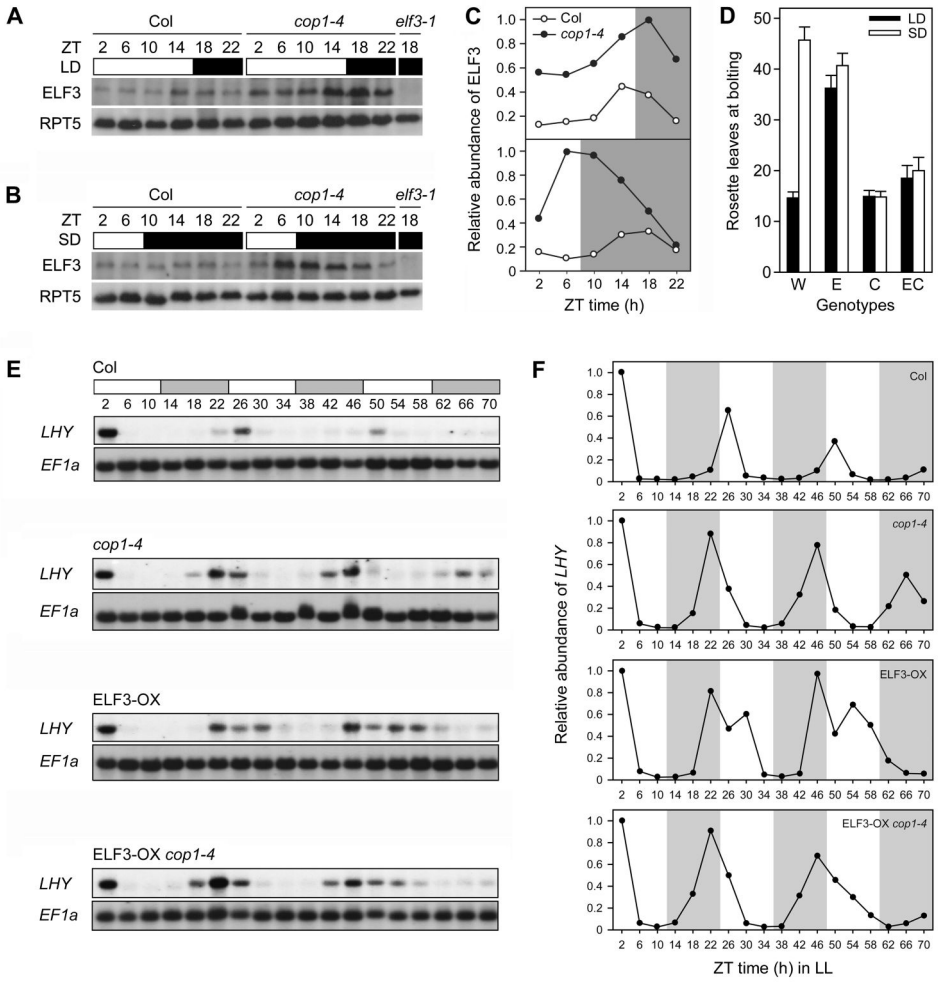

Figure 5. Rhythmic Accumulation and ELF3 Function are Controlled by COP1

(A-C) Cycling and relative abundance of native ELF3 protein in WT and *cop1-4* plants under LD (A, C) and SD (B, C). Ten μg of nuclear protein extracts from 10-d-old seedlings was loaded into each lane. Nuclear protein extracts of *elf3-1* null mutant (Col) was used as negative control (A, B). Proteasome subunit RPT5 protein abundance was detected as loading control (A, B). Open and dark areas represent day and night periods, respectively.

(D) Effect of *ELF3* overexpression on flowering time under *cop1-4* mutation. Mean and standard deviation values were obtained from at least 20 plants. W, WT (Col); E, ELF3-OX; C, *cop1-4*; EC, ELF3-OX *cop1-4*.

(E–F) Periodic accumulation of *LHY* transcripts in WT, *cop1-4*, ELF3-OX and ELF3-OX *cop1-4* plants. Ten μg of total RNA from LL-entrained plants was loaded into each lane. Radiolabeled *LHY* cDNA was used as a probe. *LHY* mRNA levels are expressed relative to the abundance of *ELONGATION FACTOR 1a* (*EF1a*) transcripts (F). White and grey areas represent subjective days and nights, respectively.

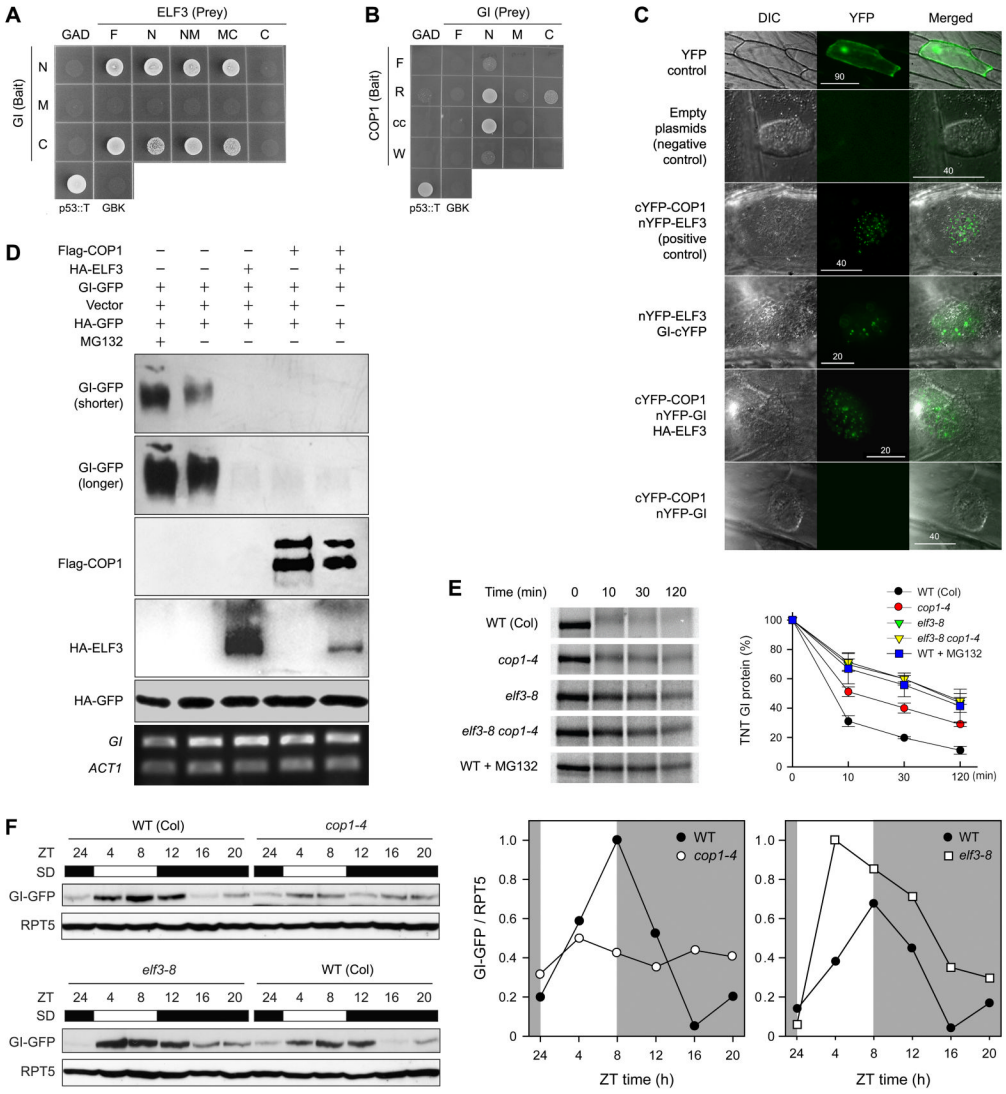

Figure 6. COP1 and ELF3 Interact with GI and Promote its Degradation in Vivo

(A) ELF3 interacts with GI in yeast two-hybrid assays. As preys, full-length ELF3 (F; aa 1–695) or ELF3 N-terminal (N; 1–261), middle (M; 261–440), C-terminal (C; 440–695), NM (1–440), and MC (261–695) regions (Liu et al., 2001). For baits, GI was divided by three parts, such as N-terminal (N; 1–507), middle (M; 401–907), and C-terminal (C; 801–1173). p53::T indicates a positive control (Clontech). Empty pGBK (bait) and pGAD (prey) plasmids were used as negative controls.

(B) RING-finger and coiled-coil domains of COP1 interact with N-terminal region of GI in yeast two-hybrid assays. Full-length and three domains of COP1 were used as baits (see Figure 4B), and full-length (1–1173) and three parts of GI in (A) as preys. R, RING-finger; CC, coiled-coil; W, WD40 repeat domains of COP1.

(C) BiFC visualization of COP1-ELF3, ELF3-GI and COP1-GI interactions in the nucleus of onion epidermal cells. Empty BiFC plasmids were used as a negative control. For COP1-ELF3 (positive control) and ELF3-GI interactions, two BiFC constructs encoding the indicated partial-YFP fusions were co-bombarded into cell layers. For COP1-GI interaction, HA-ELF3-expressing plasmids were co-bombarded with the cYFP-COP1 and nYFP-GI constructs. In all cases, YFP signals were only detectable in the presence of MG132 (50 μM) and upon dark

incubation. Transient expression of nYFP-COP1 and cYFP-ELF3, cYFP-ELF3 and GI-nYFP, and nYFP-COP1, cYFP-GI with or without HA-ELF3 showed the same results (data not shown). These experiments were repeated at least three times with similar results. Numbers in bars =μm. DIC, differential interference contrast.

(D) GI accumulation is controlled by COP1 and ELF3 in a proteasome-dependent manner. Immunoblots of protein extracts corresponding to agro-infiltrated *N. benthamiana* leaves with indicated plasmids in the presence or absence of MG132 (25 μM). GI-GFP (two upper panels, corresponding to short and long immunoblot exposures), Flag-COP1, and HA-ELF3 and HA-GFP (input control) were detected using anti-GFP, anti-Flag and anti-HA antibodies, respectively. *GI-GFP* (*GI*) and *ACTIN1* (*ACT1*) mRNA expression levels in agro-infiltrated leaves were analyzed by competitive RT-PCR (bottom panel).

(E) Degradation of ^{35}S-labelled GI (TNT GI) after incubation for the indicated times (min) with cellular extracts from WT (Col), MG132-treated WT (WT+MG132), *cop1-4*, *elf3-8* or *elf3-8 cop1-4* plants grown under LD and harvested at ZT22. Mean and standard deviation values of three replicates are shown.

(F) Cycling and relative abundance of GI-GFP protein in WT, *cop1-4* and *elf3-8* plants grown under SD. Total protein extracts (100 μg) from 20-d-old seedlings were loaded into each lane. Anti-GFP antibody was used to detect GI-GFP. Proteasome subunit RPT5 protein abundance was detected as loading control. Open and dark areas represent day and night periods, respectively.

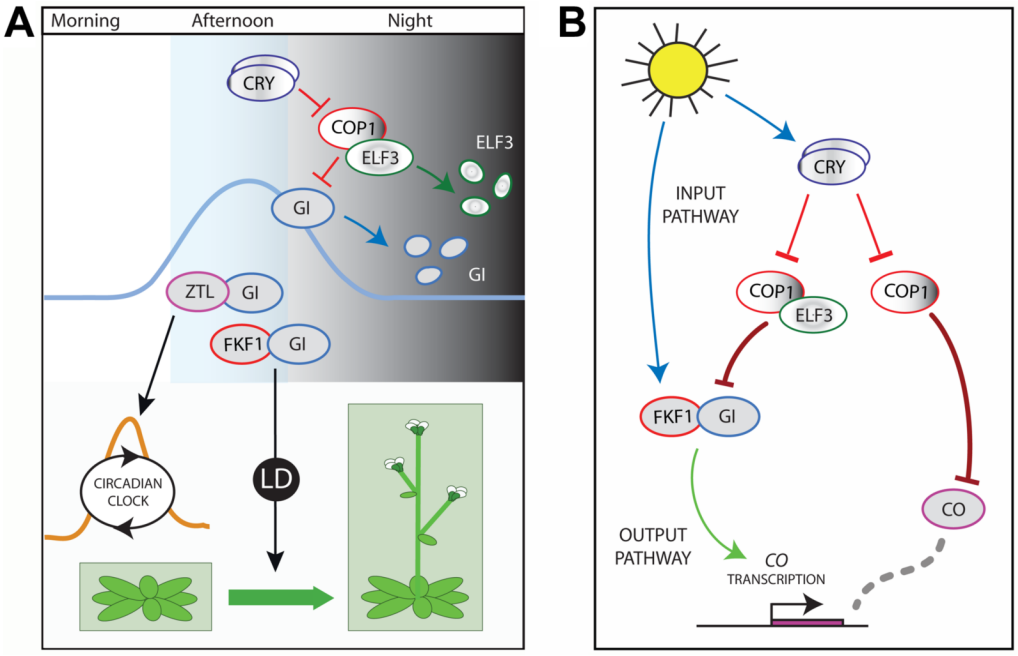

Figure 7. Model of COP1 and ELF3 Control of Flowering Time and Circadian Function

(A) COP1 and ELF3 coordinately regulate flowering time and circadian rhythms by modulating the biological activity of GI on light-input signaling to the circadian clock. At night, COP1 and ELF3 highly accumulate in the nucleus, where they interact and bind to GI to promote its degradation. In this process, ELF3 may act as a substrate adaptor to allow COP1-GI interaction, which also results in COP1-mediated degradation of ELF3, possibly to limit the extent of ELF3 function. Dark-driven GI destabilization mediated by COP1 and ELF3 plays an antagonistic role to blue-light enhanced formation of ZTL-GI and FKF1-GI complexes, whose cyclic accumulation is essential for proper clock oscillation and timely photoperiodic flowering, respectively (Kim et al., 2007; Sawa et al., 2007). By inhibiting COP1 activity, CRY may help to stabilize GI, having thus an overlapping function with blue-light sensing ZTL/FKF1/LKP2 proteins.

(B) COP1 roles in the control of flowering time involve regulation of CO function at both transcriptional and post-translational levels. Regulation of GI stability allows COP1 and ELF3 to modulate expression of floral inducer *CO*, a gene positively controlled by the FKF1-GI complex. Additionally, COP1 mediates degradation of CO, mainly at night, limiting thus its function in the promotion of flowering in response to seasonal changes in photoperiod.