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Interaction of CCA1 and ELF3

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Title:
CCA1 and ELF3 Interact in the Control of Hypocotyl Length and Flowering Time in Arabidopsis

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Abstract

The circadian clock is an endogenous oscillator with a period of ~24 hours that allows organisms to anticipate, and respond to, changes in the environment. In Arabidopsis (Arabidopsis thaliana), the circadian clock regulates a wide variety of physiological processes including hypocotyl elongation and flowering time. CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) is a central clock component, and CCA1 overexpression causes circadian dysfunction, elongated hypocotyls and late flowering. EARLY FLOWERING 3 (ELF3) modulates light input to the clock and is also postulated to be part of the clock mechanism. elf3 mutations cause light-dependent arrhythmicity, elongated hypocotyls and early flowering. Although both genes affect similar processes, their relationship is not clear. Here we show that CCA1 represses ELF3 by associating with its promoter, completing a CCA1-ELF3 negative feedback loop that places ELF3 within the oscillator. We also show that ELF3 acts downstream of CCA1, mediating the repression of PHYTOCHROME-INTERACTING FACTORs 4 and 5 in the control of hypocotyl elongation. In the regulation of flowering, our findings show that ELF3 and CCA1 either cooperate or act in parallel through the CONSTANS/FLOWERING LOCUS T (FT) pathway. In addition, we show that CCA1 represses GIGANTEA and SUPPRESSOR OF CONSTANS 1 by direct interaction with their promoters, revealing additional connections between the circadian clock and the flowering pathways.
The circadian clock is an endogenous timekeeper that generates rhythms of ~24 hours in biological processes. Environmental signals set the pace of the clock, which then drive self-sustaining oscillations. Conceptually, the Arabidopsis (*Arabidopsis thaliana*) clock can be divided into inputs such as light and temperature, a core oscillator, and outputs including the physiological processes of leaf movement, hypocotyl elongation, and photoperiodic flowering (Johnson et al., 1998; Dowson-Day and Millar, 1999).

The core oscillator consists of three interlocking feedback loops, designated as morning, central and evening loops (Locke et al., 2005; Locke et al., 2006; Zeilinger et al., 2006; Lu and Tobin, 2011). The central loop contains CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), two homologous Myb-domain transcription factors whose functions partially overlap (Schaffer et al., 1998; Wang and Tobin, 1998; Lu et al., 2009). CCA1 and LHY accumulate at dawn to repress the evening-expressed TIMING OF CAB EXPRESSION 1 (TOC1) by binding to its promoter (Strayer et al., 2000; Alabadi et al., 2001). In the morning loop, CCA1 and LHY promote the expression of *PSEUDORESPONSE REGULATORS* 9 (*PRR9*) and 7 (*PRR7*), which repress the expression of CCA1 and LHY (Farre et al., 2005; Nakamichi et al., 2010). EARLY FLOWERING 3 (ELF3) accumulates in the evening and has dual roles in the clock; it modulates light input and operates as a core clock component to repress *PRR9* (Hicks et al., 1996; McWatters et al., 2000; Covington et al., 2001; Thines and Harmon, 2010; Dixon et al., 2011; Kolmos et al., 2011). Other factors which act close to or within the central oscillator include *PRR3, PRR5, GIGANTEA (GI), CCA1 HIKING EXPEDITION, LUX ARRHYTHMO (LUX)/PHYTOCLOCK 1, and EARLY FLOWERING 4 (ELF4)* (Doyle et al., 2002;
Hazen et al., 2005; Onai and Ishiura, 2005; Kim et al., 2007; Para et al., 2007; Nakamichi et al., 2010; Thines and Harmon, 2010; Dixon et al., 2011; Helfer et al., 2011).

Hypocotyl elongation is a physiological response that is controlled by both the clock and light signaling. The circadian clock regulates the expression of two growth-promoting transcription factors PHYTOCHROME-INTERACTING FACTORS 4 (PIF4) and 5 (PIF5) through a complex of the evening expressed proteins ELF3, ELF4, and LUX (Nusinow et al., 2011). This evening complex represses PIF4 and PIF5 during the first half of the night by binding to their promoters. Later in the night, PIF4 and PIF5 mRNA levels increase and hypocotyl elongation occurs. At dawn, PIF4 and PIF5 are targeted for degradation through their interaction with the light-activated form of phytochrome B (Huq and Quail, 2002; Khanna et al., 2004). In elf3-1, the levels of PIF4 and PIF5 mRNA are elevated compared with wild type (WT), particularly during the early evening (Nusinow et al., 2011). In CCA1-OX plants, PIF4 and PIF5 cycle with reduced amplitudes and levels of transcript are high throughout the night, giving rise to long hypocotyls (Nozue et al., 2007; Niwa et al., 2009).

The timing of flowering in Arabidopsis is controlled by the autonomous, vernalization, gibberellin, and photoperiodic pathways (Corbesier and Coupland, 2006; Fornara et al., 2010; Srikanth and Schmid, 2011). There are multiple points of crosstalk between these pathways. For example, FLOWERING LOCUS C (FLC) operates in the autonomous and vernalization pathways (Michaels and Amasino, 1999, 2001) and represses FLOWERING LOCUS T (FT) and SUPPRESSOR OF CONSTANS 1 (SOC1), whose corresponding proteins are floral integrators (Corbesier and Coupland, 2006). The circadian clock controls photoperiodic flowering chiefly through the GI-CONSTANS
GI interacts with FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 during the day to promote CO expression through degradation of the CO repressor CYCLING DOF FACTOR 1 (Imaizumi et al., 2003; Imaizumi et al., 2005; Sawa et al., 2007). CO is stabilized in long days, FT expression is induced, and flowering occurs (Samach et al., 2000; Suarez-Lopez et al., 2001).

CCA1-OX plants exhibit long hypocotyls, late flowering and widespread arrhythmicity (Wang and Tobin, 1998). Light-dependent arrhythmicity, elongated hypocotyls and early flowering result from elf3 mutations (Zagotta et al., 1996). Although CCA1 and ELF3 overexpressors and mutants are well characterized, how CCA1 and ELF3 interact genetically in the circadian oscillator and in the control of the physiological processes of hypocotyl elongation and flowering has not been elucidated. In this study, we show that CCA1 represses ELF3 expression through association with its promoter to close a loop that includes PRR9. We have determined that ELF3 acts downstream of CCA1 in hypocotyl elongation and that PIF4 and PIF5 are not the only factors involved in this process. In the control of flowering time, we have shown that ELF3 and CCA1 either cooperate or act in parallel through the CO-FT pathway. Finally, our results show that CCA1 binds directly to the GI and SOC1 promoters to repress their expression, providing proof of a new connection between the central and evening loops of the Arabidopsis oscillator, and introducing additional links between CCA1 and the flowering pathways.

RESULTS
ELF3 and CCA1 Form a Negative Feedback Loop

To determine how ELF3 affects CCA1 expression, CCA1 mRNA levels were examined over a time course in ELF3-OX and elf3-1 plants by qRT-PCR (Fig. 1A). Constitutive overexpression of ELF3 resulted in slightly elevated peak levels of CCA1 mRNA, whereas the peak levels of CCA1 mRNA were reduced in elf3-1 mutant plants. This result is consistent with previous reports that ELF3 represses PRR9 and that PRR9 represses CCA1 expression (Nakamichi et al., 2010; Dixon et al., 2011). Therefore, ELF3 positively regulates the expression of CCA1. To determine whether CCA1 forms a feedback loop with ELF3, we examined ELF3 expression in CCA1-OX and cca1-1 plants (Fig. 1B). In CCA1-OX plants, ELF3 was repressed and its circadian rhythms abolished. This result is consistent with previous reports showing that overexpression of CCA1 results in arrhythmicity (Wang and Tobin, 1998). In cca1-1, ELF3 expression was slightly elevated, but still exhibited circadian rhythms with evening peaks of mRNA; maintenance of circadian rhythms in cca1-1 plants has been reported previously (Green and Tobin, 1999). Taken together, these results show that CCA1 represses the expression of ELF3. Therefore, ELF3 and CCA1 form a negative feedback loop through PRR9.

CCA1 Represses ELF3 by Interacting with its Promoter

The ELF3 gene contains a CCA1-binding site (CBS) between -248 and -241 relative to its transcription start site (Fig. 2A) (Wang et al., 1997). To examine whether the effect of CCA1 on ELF3 expression was by direct association with its promoter,
chromatin immunoprecipitation (ChIP) was performed using anti-CCA1 antibody. Figure 2B demonstrates that an anti-CCA1 antibody efficiently immunoprecipitated a fragment of ELF3 that contains the CBS but not another one located in the downstream coding region, suggesting that CCA1 binds directly to the promoter of ELF3. If this association results in repression, a pulse of CCA1 using an ethanol-inducible system (Knowles et al., 2008) should result in a reduction of ELF3 mRNA shortly after the induction of CCA1 protein. Figure 2D shows that a pulse of CCA1 causes a reduction in ELF3 expression within one hour (Fig. 2C), suggesting that CCA1 binds directly to the ELF3 promoter to repress its expression. In WT Arabidopsis, CCA1 association with the ELF3 promoter was only observed at two hours after dawn (ZT2) when CCA1 protein is most abundant, but not at two hours after dusk (ZT14) when CCA1 protein is least abundant (Fig. 2B). These results are consistent with CCA1 repression of ELF3 during the day and rising of ELF3 expression in the evening when CCA1 is absent.

Interactions between CCA1 and ELF3 in the Control of Hypocotyl Length

To determine whether ELF3 and CCA1 interact genetically in the regulation of hypocotyl length, we compared hypocotyl lengths of elf3-1, CCA1-OX and elf3-1 CCA1-OX plants. Under both LD and SD conditions, elf3-1 and CCA1-OX have elongated hypocotyls (Fig. 3) as previously reported (Zagotta et al., 1996; Kim et al., 2005). The hypocotyl length of elf3-1 CCA1-OX plants was more similar to the hypocotyl length of CCA1-OX than the elf3-1 mutant, which has shorter hypocotyls than CCA1-OX seedlings (Fig. 3), suggesting that additional factors downstream of CCA1 parallel to ELF3 could
be involved in hypocotyl elongation. It is known that the circadian clock can affect hypocotyl elongation through its regulation of PIF4 and PIF5 (Nozue et al., 2007; Nusinow et al., 2011). To determine the mechanism that gives rise to the observed long hypocotyls in elf3-1, CCA1-OX and elf3-1 CCA1-OX plants, we examined the expression of PIF4 and PIF5. In both LD and SD, PIF4 and PIF5 expression was higher in elf3-1, CCA1-OX and elf3-1 CCA1-OX plants than in WT in the dark (Fig. 4, A-D). However, there were no appreciable differences in PIF4 and PIF5 expression when elf3-1, CCA1-OX and elf3-1 CCA1-OX plants were compared, suggesting that other factors contribute to their differing hypocotyl lengths.

**Interactions between CCA1 and ELF3 in the Control of Flowering Time**

We determined whether ELF3 and CCA1 interact genetically to control photoperiodic flowering by measuring flowering time and examining the expression of the flowering genes FT, SOC1, FLC, GI, and CO. Under LD growth conditions, elf3-1 CCA1-OX plants flowered before CCA1-OX plants, but later than elf3-1 and WT plants (Fig. 5, A-C), suggesting that ELF3 acts mainly upstream of CCA1, but can also act coordinately or in parallel with CCA1. There were no significant differences in FLC expression levels among WT, elf3-1, CCA1-OX, and elf3-1 CCA1-OX plants (Fig. 6C), suggesting that neither CCA1 nor ELF3 are involved in the autonomous and vernalization pathways. In the elf3-1 plants, FT, GI and CO were derepressed compared with WT (Fig. 6, A, D, and E) as previously reported (Kim et al., 2005). CCA1-OX plants exhibited repressed FT, SOC1, GI, and CO expression (Fig. 6, A, B, D, and E), which is in
accordance with its late flowering phenotype (Fig. 5). The expression levels of FT, SOC1, GI, and CO were similar in CCA1-OX and elf3-1 CCA1-OX plants, and low compared to WT and elf3-1 plants (Fig. 6, A, B, D, and E). These results suggest that in LD, ELF3 acts mainly upstream of CCA1 in the control of the expression of FT, SOC1, GI, and CO.

Arabidopsis is a facultative long day plant, and it flowers much later in SD than in LD. Constitutive overexpression of CCA1 delays flowering in SD, although the effect is not as dramatic as in LD (Fig. 5), and the elf3-1 mutation causes significantly earlier flowering in SD (Fig. 5) (Hicks et al., 1996; Hicks et al., 2001). Similar to in LD, elf3-1 CCA1-OX plants flowered earlier than CCA1-OX plants, but later than the elf3-1 mutant in SD. When compared to WT, elf3-1 CCA1-OX plants flowered later, but with fewer rosette leaves. In the analysis of flowering gene expression, we obtained similar results to LD for SOC1, FLC, GI, and CO (Fig. 7, B-E), suggesting that like in LD, ELF3 operates upstream of CCA1 in the control of their gene expression in SD. However, the pattern of FT expression in SD was different. In elf3-1 CCA1-OX plants, FT expression was intermediate between that of elf3-1 and CCA1-OX plants (Fig. 7A). Therefore, ELF3 and CCA1 act either in parallel, or coordinately, to control FT expression in SD.

CCA1 Represses GI and SOC1 by Associating with their Promoters

The expression of GI and SOC1 were significantly repressed in CCA1-OX (Fig. 6B, 6E, 7B and 7E) and the promoters of GI and SOC1 both contain CBSs (Figs. 8A and 9A) (Wang et al., 1997). To examine whether the effect of CCA1 on GI and SOC1 expression was by direct association with their promoter in planta, we performed ChIP in WT.
CCA1-OX and cca1-1 lines. Figures 8B and 9B show that at ZT2, the fragments of GI and SOC1 containing CBSs were significantly enriched in WT and CCA1-OX plants, but not in cca1-1 plants, suggesting that CCA1 binds to the GI and SOC1 promoters in Arabidopsis. In addition, an ethanol-induced pulse of CCA1 was able to reduce GI and SOC1 expression within one hour (Figs. 8D and 9D), whereas ethanol treatment of a control line produced no changes in their expression (Figs. 8C and 9C). These results establish that repression of GI and SOC1 is due to the direct association of CCA1 with their promoters.

DISCUSSION

It has been shown that ELF3 associates with the promoter of PRR9 to repress its expression and that PRR9 represses CCA1 by interaction with its promoter (Nakamichi et al., 2010; Dixon et al., 2011). Our data that CCA1 represses ELF3 expression by association with its promoter (Figs. 1 and 2) close the negative feedback loop composed of ELF3, PRR9 and CCA1 (Fig. 10). These results support the idea that ELF3 is a component of the oscillator itself (Thines and Harmon, 2010; Dixon et al., 2011) in addition to its established role as a modulator of light input to the clock (Hicks et al., 1996; McWatters et al., 2000; Covington et al., 2001; Kolmos et al., 2011). We also show that ELF3 acts downstream of CCA1 to mediate the repression of PIF4 and PIF5 in the control of hypocotyl elongation (Figs 3 and 4). In the regulation of flowering, ELF3 and CCA1 act in a coordinated manner or in parallel pathways (Figs. 5-7). Finally, we have
shown that CCA1 represses GI and SOC1 by association with their promoters (Figs. 8 and 9), revealing additional connections between CCA1 and the flowering pathways.

Hypocotyl elongation is controlled by many factors, including the convergence of light and clock signaling (Nozue et al., 2007). A recent report has shown that ELF3 forms a complex with ELF4 and LUX to directly repress PIF4 and PIF5 expression and an elf3 pif4 pif5 triple mutant exhibits hypocotyls of similar length to WT plants (Nusinow et al., 2011). In this study, we found that ELF3 expression is repressed directly by CCA1, and thus ELF3 acts downstream of CCA1 to repress PIF4 and PIF5. However, PIF4 and PIF5 RNA levels were elevated to similar levels in elf3-1, elf3-1 CCA1-OX, and CCA1-OX plants while elf3-1 showed a weaker hypocotyl phenotype than CCA1-OX and elf3-1 CCA1-OX plants. This is consistent with CCA1 affecting additional factors besides ELF3, PIF4 and PIF5 in controlling hypocotyl elongation (Hazen et al., 2005; Li et al., 2011).

The circadian clock regulates flowering through the photoperiodic pathway that includes CO and FT. Both ELF3 and CCA1 act as negative regulators upstream of CO and FT, but their relationship in controlling the photoperiodic pathway is unclear. Under both LD and SD conditions, elf3-1 CCA1-OX flowers before CCA1-OX plants, but after elf3-1 plants (Fig. 5), suggesting that ELF3 and CCA1 do not act in a linear pathway. In LD, expression of CO and FT were high in the elf3-1 mutant, but low in elf3-1 CCA1-OX and CCA1-OX plants (Fig.6, A and D), suggesting that ELF3 could act upstream of CCA1 in the control of CO and FT. In SD, CO and FT levels are high in elf3-1, but low in CCA1-OX plants (Fig. 7, A and D). In elf3-1 CCA1-OX plants, high levels of FT message were observed, but these levels were not in accordance with the low level of CO expression (Fig. 7, A and D). This result is in agreement with a previous report showing that ELF3
can act independently of CO to control flowering time (Kim et al., 2005). It has been shown that ELF3 represses *GI* transcription independently of CCA1, possibly through its repression of *PRR9* (Dixon et al., 2011), and ELF3 protein is involved in facilitating the interaction between CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) and *GI*. The COP1-*GI* interaction destabilizes the *GI* protein to ensure proper *GI* function in the control of photoperiodic flowering (Yu et al., 2008). In this study, we showed that CCA1 could bind directly to the *GI* promoter to repress its expression (Fig. 8), adding another link within the clock, and between the clock and the photoperiodic flowering pathway. In accordance with these data, *gi-3 cca1-1 lhy-11* triple mutants exhibit WT flowering time, even though *cca1-1 lhy-11* double mutants flower very early (Mizoguchi et al., 2005); this result suggests that CCA1 acts on flowering time through *GI*. It is possible that ELF3 and CCA1 function in the photoperiodic flowering pathway converges on their regulation of *GI*. Recent reports have shown that *GI* can activate flowering through a CO-independent pathway through three possible mechanisms. First, *GI* regulates miR172, which represses genes such as *TARGET OF EAT 1* and *SCHLAFMUTZE* post-transcriptionally, relieving their repression of *FT* (Jung et al., 2007). Second, *GI* can interact with *FT* repressors such as SHORT VEGETATIVE PHASE, TEMPRANILLO (TEM) 1 and TEM2 to regulate the stability of *FT* repressors or their accessibility to the *FT* promoter (Sawa and Kay, 2011). Third, *GI* interacts physically with the *FT* promoter either alone or in a complex with another protein (Sawa and Kay, 2011). The strong CO-independent *FT* induction in *elf3-1 CCA1-OX* plants under SD conditions is likely due to the fact that the flowering pathways form a highly integrated network and CO-dependent *FT* activation is probably more prominent in LD. We have presented a model in which
ELF3 and CCA1 independently regulate the expression of GI, and GI regulates the expression of FT through both CO-dependent and -independent pathways (Fig.10).

The MADS box transcription factor SOC1 is a key floral activator integrating multiple flowering pathways. The expression of SOC1 is positively regulated by FT (Yoo et al., 2005) and negatively regulated by FLC, which forms a floral repressor complex with other proteins and directly binds to the promoter of SOC1 (Helliwell et al., 2006). In this study, we found that elf3-1 CCA1-OX plants exhibit low levels of SOC1 message, similar to CCA1-OX plants (Fig. 6B and 7B), indicating that CCA1 acts downstream of ELF3 to negatively regulate the expression of SOC1. This result is in accordance with a previous report showing that SOC1 expression is higher in cca1-1 lhy-11 double mutants (Fujiwara et al., 2005). Furthermore, we demonstrated that CCA1 significantly represses SOC1 expression through direct interaction with its promoter (Fig. 9), illustrating a novel way that the circadian clock controls flowering, through direct regulation of a floral integrator (Fig. 10).

The examination of flowering gene expression revealed that neither ELF3 nor CCA1, participate in the regulation of FLC (Fig. 6C and 7C). However, the elf3-8 mutant exhibits reduced FLC expression in a photoperiod-independent manner, suggesting that ELF3 can affect FLC expression (Yu et al., 2008). In elf3-8, there is a base pair change at the exon 4 splice acceptor site, resulting in a truncated protein that includes 28 amino acids from a different frame before a premature stop codon (Hicks et al., 2001). In this study, we utilized the elf3-1 mutant which produces a truncated protein resulting from a single base pair change that gives rise to an early stop codon (Hicks et al., 1996). A detailed comparison between these two mutants has not been carried out, but they do
appear to flower at the same time under LD and SD (Liu et al., 2001; Kim et al., 2005; Yu et al., 2008; Nefissi et al., 2011). \( elf3-7 \) is a weak allele which contains a single base change at the exon 1 splice donor site (Hicks et al., 2001). Through the use of cryptic splice sites, the \( elf3-7 \) mutant can give rise to a combination of truncated ELF3 proteins, or a version that is missing eight amino acids (Hicks et al., 2001). A recent report showed that \( elf3-7 \) has less severe hypocotyl and flowering phenotypes than \( elf3-1 \), but it has the same effect on \( CCA1 \) and \( LHY \) expression (Kolmos et al., 2011), showing that it is difficult to predict how a given mutant allele of \( ELF3 \) will behave. Although \( elf3-1 \) and \( elf3-8 \) mutants have similar flowering times, how they act on the various flowering genes, including \( FLC \), may be different because a complete understanding of the functions of ELF3 protein domains is lacking.

In summary, our study of the interplay between the central clock component \( CCA1 \) and ELF3 has completed the \( CCA1 \)-Elf3 negative feedback loop that places Elf3 within the oscillator. We have shown that \( CCA1 \) represses \( GI \) and \( SOC1 \) expression by direct association with their promoters, not only highlighting the multiple roles of \( CCA1 \) in the regulation of flowering time, but also revealing additional connections between the circadian clock and the flowering pathway.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**
Arabidopsis (Arabidopsis thaliana; Columbia ecotype) was used for all experiments unless stated otherwise. The elf3-1 mutant (Zagotta et al., 1996), ELF3 overexpression line (ELF3-OX) (Covington et al., 2001), the cca1-1 mutant (Green and Tobin, 1999) and CCA1 overexpression line (CCA1-OX, line o38) (Wang and Tobin, 1998) are as described previously. To achieve overexpression of CCA1 in the elf3-1 background, the CCA1-OX transgenic line was crossed with the elf3-1 mutant and plants homozygous for both the 35S::CCA1 transgene and the elf3-1 mutation were identified as previously described (Wang and Tobin, 1998; Kim et al., 2005). Seedlings were grown under a 12 h fluorescent light (100 µmol m^{-2} s^{-1})/12 h dark (12L/12D) photoperiod at a constant temperature of 22°C, unless otherwise stated.

Measurement of Hypocotyl Length and Flowering Time

Arabidopsis plants were grown on soil under either LD (16 h light/8 h dark) or SD (8 h light/16 h dark) conditions with 100 µmol m^{-2} s^{-1} white fluorescent light. Hypocotyl length was measured from days five to seven. Flowering time was scored by counting the number of days to, and number of rosette leaves at, flowering.

RNA Extraction and qRT-PCR

1-2 week-old seedlings were grown on Murashige and Skoog medium (Murashige and Skoog, 1962) with 1.5% agar. For the circadian experiments, samples were collected every 4 h either during the light/dark cycle or in continuous white light. Total RNA
extraction and qRT-PCR were carried out as previously described (Lu et al., 2011). 

Actin2 was used as a non-cycling reference, and the expression levels were normalized to the control. The primers used for amplification are listed in Supplemental Table S1.

ChIP

ChIP was performed as previously described (Lu et al., 2011) using affinity-purified anti-CCA1 antibody (Wang and Tobin, 1998) for immunoprecipitation. The primers used for amplification are listed in Supplemental Table S1.

Ethanol Pulse

EtOH treatment was performed as previously described (Knowles et al., 2008). Control plants contained the regulator construct 35S::AlcR::T\textsubscript{NOS}. 10 day-old seedlings were treated with 10 min of EtOH vapor of 1% (v/v) EtOH. Samples were collected at the time of induction (0 h), 1 h, 2 h, and 4 h after EtOH treatment.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative data library using the following accession numbers: CCA1 (At2g46830), ELF3 (At2g25930), PIF4 (At2g43010), PIF5 (At3g59060), FT (At1g65480), CO (At5g15840), FLC (At5g10140), SOC1 (At2g45660), GI (At1g22770) and ACT2 (At3g18780).
Supplemental Data

Supplemental Table S1. List of PCR primer sequences

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FIGURE LEGENDS
Figure 1. CCA1 and ELF3 form a negative feedback loop. A, CCA1 expression in WT, elf3-1 and ELF3-OX plants under diurnal and continuous light (LL) conditions. B, ELF3 expression in WT, cca1-1 and CCA1-OX plants under diurnal and LL conditions. 10 day-old seedlings sampled at 4 h intervals were analyzed by qRT-PCR. Day, night and subjective night are denoted by white, black and hatched bars, respectively. The data are presented as the mean of two biological replicates ± SD. All experiments were done at least twice with similar results.

Figure 2. CCA1 binds directly to the promoter of ELF3 to repress its expression. A, Schematic drawing of genomic ELF3 and the regions examined by ChIP. Exons are represented by black boxes, untranslated regions are represented by gray boxes and introns are represented by lines. CBS (“C”) are represented by open boxes. B, Binding of CCA1 to the ELF3 promoter in vivo. ChIP assays were performed with WT (Col), cca1-1 and OX (CCA1-OX) seedlings collected at ZT2 and ZT14. qRT-PCR was performed on the precipitates; ELF3-P amplifies the region that contains a CBS and ELF3-N amplifies a region in the downstream coding region. Results were normalized to the input DNA. C and D, A pulse of CCA1 expression represses the accumulation of ELF3 RNA. Control (C) and Alc::CCA1 (D) seedlings were given an EtOH pulse at 32 h in LL (CT-8). qRT-PCR analysis of ELF3 expression at the time of induction (0 h), 1 h, 2 h and 4 h after EtOH treatment are represented. Diamonds, no EtOH treatment; squares, 1% EtOH. The mean of two biological replicates ± SD is shown. All of these experiments were done at least twice with similar results.
Figure 3. Constitutive overexpression of CCA1 protein affects hypocotyl growth in LD and SD. The WT (circles), elf3-1 (squares), CCA1-OX (diamonds) and elf3-1 CCA1-OX (triangles) plants were grown for 5 to 7 d under LD (16L/8D) conditions (A) or under SD (8L/16D) conditions (B). Average hypocotyl lengths ± SD (n = 20) are presented. All experiments were done at least twice with similar results.

Figure 4. Transcript abundance of PIF4 and PIF5 in LD and SD. Expression levels of PIF4 (A and C) and PIF5 (B and D) transcripts in WT (diamonds), elf3-1 (squares), CCA1-OX (triangles) and elf3-1 CCA1-OX (circles) plants. 8 day-old seedlings grown under LD (16L/8D) conditions (A and B) or 12 day-old seedlings grown under SD (8L/16D) conditions (C and D) sampled at 4 h intervals were analyzed by qRT-PCR. Day and night are denoted by white and black bars, respectively. The data are presented as the mean of two biological replicates ± SD. All experiments were done at least twice with similar results.

Figure 5. Constitutive overexpression of CCA1 protein delays flowering in LD and SD. A, Photographs of plants of various genotypes grown for 25 days under LD (16L/8D) conditions. B and C, Flowering time of seedlings of various genotypes under LD (16L/8D) conditions. D, Photographs of plants of various genotypes grown for 50 days under SD (8L/16D) conditions. E and F, Flowering time of seedlings of various genotypes under SD (8L/16D) conditions. Flowering time is expressed as either days to bolting or rosette leaf number. Data are means ± SD (n = 18-25).
**Figure 6.** Transcript abundance of flowering time genes under LD (16L/8D) conditions. Expression levels of *FT* (A), *SOC1* (B), *FLC* (C), *CO* (D) and *GI* (E) transcripts in WT (diamonds), *elf3-1* (squares), *CCA1*-*OX* (triangles) and *elf3-1 CCA1*-*OX* (circles) plants. 8 day-old seedlings grown under LD (16L/8D) conditions sampled at 4 h intervals were analyzed by qRT-PCR. Day and night are denoted by white and black bars, respectively. The data are presented as the mean of two biological replicates ± SD. All experiments were done at least twice with similar results.

**Figure 7.** Transcript abundance of flowering time genes under SD (8L/16D) conditions. Expression levels of *FT* (A), *SOC1* (B), *FLC* (C), *CO* (D) and *GI* (E) transcripts in WT (diamonds), *elf3-1* (squares), *CCA1*-*OX* (triangles) and *elf3-1 CCA1*-*OX* (circles) plants. 12 day-old seedlings grown under SD (8L/16D) conditions sampled at 4 h intervals were analyzed by qRT-PCR. Day and night are denoted by white and black bars, respectively. The data are presented as the mean of two biological replicates ± SD. All experiments were done at least twice with similar results.

**Figure 8.** CCA1 binds directly to the promoter of *GI* to repress its expression. A, Schematic drawing of genomic *GI* and the regions examined by ChIP. Exons are represented by black boxes, untranslated regions are represented by gray boxes and introns are represented by lines. CBS (“C”) are represented by open boxes. B, Binding of CCA1 to the *GI* promoter in vivo. ChIP assays were performed with WT (Col), *cca1-1* and OX (*CCA1*-*OX*) seedlings collected at ZT2 and ZT14. qRT-PCR was performed on the precipitates; GI-P amplifies the region that contains a CBS and GI-N amplifies a
region in the downstream coding region. Results were normalized to the input DNA. C and D, A pulse of CCA1 expression represses the accumulation of GI RNA. Control (C) and Alc::CCA1 (D) seedlings were given an EtOH pulse at 32 h in LL (CT-8). qRT-PCR analysis of GI expression at the time of induction (0 h), 1 h, 2 h and 4 h after EtOH treatment are represented. Diamonds, no EtOH treatment; squares, 1% EtOH. The mean of two biological replicates ± SD is shown. All of these experiments were done at least twice with similar results.

Figure 9. CCA1 binds directly to the promoter of SOC1 to repress its expression. A, Schematic drawing of genomic SOC1 and the regions examined by ChIP. Exons are represented by black boxes, untranslated regions are represented by gray boxes and introns are represented by lines. CBS (“C”) are represented by open boxes. B, Binding of CCA1 to the SOC1 promoter in vivo. ChIP assays were performed with WT (Col), cca1-1 and OX (CCA1-OX) seedlings collected at ZT2 and ZT14. qRT-PCR was performed on the precipitates; SOC1-P amplifies the region that contains a CBS and SOC1-N amplifies a region in the downstream coding region. Results were normalized to the input DNA. C and D, A pulse of CCA1 expression represses the accumulation of SOC1 RNA. Control (C) and Alc::CCA1 (D) seedlings were given an EtOH pulse at 32 h in LL (CT-8). qRT-PCR analysis of SOC1 expression at the time of induction (0 h), 1 h, 2 h and 4 h after EtOH treatment are represented. Diamonds, no EtOH treatment; squares, 1% EtOH. The mean of two biological replicates ± SD is shown. All of these experiments were done at least twice with similar results.
Figure 10. A proposed model for how the clock-associated factors CCA1, ELF3 and PRR9 affect the expression of members of the photoperiodic flowering pathway. Solid lines indicate direct regulation. Connections that were confirmed in this study are represented by thick lines. Dotted lines indicate indirect regulation (i.e. additional steps and/or components are involved but not shown here).
Figure A:

- **WT**
- **elf3-1**
- **ELF3-OX**

**CCA1/Actin**

0.20
0.15
0.10
0.05
0.00

Time (h)

0 24 48 72 96 120

Figure B:

- **WT**
- **cca1-1**
- **CCA1-OX**

**ELF3/Actin**

0.25
0.20
0.15
0.10
0.05
0.00

Time (h)

0 24 48 72 96 120
