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J Biol Rhythms 2008; 23; 463
DOI: 10.1177/0748730408326749

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Testing Time: Can Ethanol-Induced Pulses of Proposed Oscillator Components Phase Shift Rhythms in *Arabidopsis*?

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Abstract Circadian rhythms are generated by endogenous central oscillators that respond to input from the environment and regulate rhythmic outputs. In *Arabidopsis*, more than a dozen components that affect rhythms have been identified and used to propose models of the central oscillator. However, none has been shown to fulfill one of the expected characteristics of an oscillator component: that a pulse of its expression shifts the phase of circadian rhythms. Here we show that a pulse of the proposed oscillator components CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) causes dramatic phase shifts in rhythms of expression of the circadian reporter *CAB2::LUC,* as well as of the clock-associated genes *TIMING OF CAB EXPRESSION 1* (*TOC1*) and *GIGANTEA* (*GI*). These results demonstrate that pulses of either CCA1 or LHY are capable of resetting the circadian clock. In contrast, a pulse of *TOC1* expression did not elicit phase shifts. Control of TOC1 protein level is in part posttranscriptional; thus a pulse of TOC1 protein could be induced only at times when it is already high. Our work also shows that the ethanol-inducible system can be useful for achieving relatively short (<8 h) pulses of gene expression in seedlings.

Key words *Arabidopsis thaliana*, circadian rhythm, central oscillator, phase shift, clock gene, ethanol-inducible system

The *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1)* gene was initially characterized as a transcription factor involved in the phytochrome regulation of a gene encoding a light-harvesting chlorophyll a/b-protein (*Lhcb*/*CAB* gene; Wang et al., 1997) and was then found to be intimately involved with circadian rhythms (Wang and Tobin, 1998). Not only do both its RNA and protein show circadian rhythms of expression, but constitutive expression of *CCA1* abolishes all examined circadian rhythms, such as those seen in leaf movement and hypocotyl growth. Constitutive *CCA1* expression also results in the repression of the endogenous *CCA1* gene, showing it functions in an autoregulatory feedback loop (Wang and Tobin, 1998). Experiments with the closely related homologue, *LATE ELONGATED HYPOCOTYL (LHY)*, gave similar results (Schaffer et al., 1998). Therefore, CCA1 and LHY have been proposed as

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JOURNAL OF BIOLOGICAL RHYTHMS, Vol. 23 No. 6, December 2008 463-471
DOI: 10.1177/0748730408326749
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central components in models of the plant circadian clock (Wang and Tobin, 1998; Schaffer et al., 1998; McClung, 2006).

The consensus model for central oscillator function in eukaryotes involves multiple feedback loops based on gene transcription and translation (Young and Kay, 2001; Dunlap and Loros, 2004). An expected characteristic of an oscillator component is that a pulse of its expression will reset the phase of rhythms (Aronson et al., 1994). This feature has been demonstrated for oscillator components in cyanobacteria (KaiC; Ishiura et al., 1998), *Neurospora* (FREQUENCY; Aronson et al., 1994), and *Drosophila* (PERIOD; Edery et al., 1994). In *Arabidopsis*, the proposed core feedback loop of the oscillator consists of CCA1 and LHY, along with TIMING OF CAB EXPRESSION 1 (TOC1) (also known as PSEUDO-RESPONSE REGULATOR 1 (PRR1; Strayer et al., 2000; Makino et al., 2002)). In this proposed loop, TOC1 acts as a positive element to promote the expression of *CCA1* and *LHY* through an unknown mechanism, while CCA1 and LHY proteins function as negative elements to repress *TOC1* transcription through direct binding to its promoter (Alabadí et al., 2001). Recently, other genes such as *GIGANTEA* (*GI), LUX ARRHYTHMO* (*LUX;* also known as *PHYTOCLOCK 1* (*PCL1*)), *EARLY FLOWERING 4* (*ELF4*), *TIME FOR COFFEE* (*TIC*), and *PRR3/5/7/9* have also been proposed to be part of the plant central oscillator (reviewed in McClung, 2008). However, the biochemical functions of the encoded proteins have remained elusive and none has been tested to see the effect of a pulse of its expression on rhythms. Here we sought to determine whether pulses of *CCA1*, *LHY*, or *TOC1* expression reset the circadian clock.

MATERIALS AND METHODS

Plant lines and growth conditions. The *Alc* lines generated in this study, as well as *toc1-4* (Hazen et al., 2005) and PRR1-OX (Makino et al., 2002), are in the Col ecotype.

Seeds were stratified for 3 days in the dark at 4 C and then sown onto MS medium (Research Products International Corp., Mt. Prospect, IL) containing 1.5% (w/v) agarose (MS1.5). Seedlings were grown on 90-mm tissue culture plates. For ethanol (EtOH)-pulse experiments the seeds were sown on either 80-mm- or 15-mm-diameter nylon net filter circles (20 mm pore size; Millipore Corp., Bedford, MA) upon MS1.5. The filter served to facilitate the transfer of grown seedlings to fresh medium after EtOH treatment (see "EtOH pulse" section). Seedlings were grown under a 12-h fluorescent light (40 mol · m^{-2} · sec^{-1}):12-h dark (12:12) photoperiod at a constant temperature of 22 C.

DNA constructs for the EtOH-inducible gene expression system. EtOH-inducible DNA constructs were derived from the binary vector pbinSRNACatN (Syngenta, Berkshire, UK). The effector construct *pAlcA::Catalase:*T_{NOS} was removed from pbinSRNACatN by *Hin*dIII digestion and discarded. The remaining vector sequence harboring the regulator construct *35S::AlcR:*T_{NOS} was subsequently religated to yield pbinSRN.

A *CCA1*-containing effector construct was generated as follows: *CCA1* (1.8 kb) was amplified from *Arabidopsis* cDNA by PCR using the primers *CCA1*-EcoRI3F, 5´-GAATTCATGGAGACAAATTCG-3´ and *CCA1*-BamHIR, 5´-GACGGATCCTCATGTGGAAGC-3´ and sequenced. The *CCA1* ORF was inserted into the *Eco*RI/*Bam*HI sites of the binary vector pEGAD (Cutler et al., 2000), creating pEGAD-CCA1. The *35S* promoter and *EGFP* sequences were subsequently removed by *Stu*I + *Eco*RI digestion and replaced with the 287-bp *AlcA* promoter sequence (*Hin*dIII/*Sal*I fragment from pbinSRNACatN) by blunt-ended ligation. Correct orientation of the promoter was confirmed by sequencing. The vector was called pEGAD-pAlcA::CCA1.

Effector constructs for *LHY* and *TOC1* were made in a similar manner to pEGAD-pAlcA::CCA1. *LHY* was cloned using the primers *LHY*-MfeIF, 5´-CAATTGATGGATACTAATACA-3´ and *LHY*-XhoIR, 5´-CTCGAGTCATGTAGAAGCTTC-3´. *TOC1* was cloned using *TOC1-F*, 5´-CTGATCATGGATTTGAACG-3´ and *TOC1-R*, 5´-GCCTTAGAGACAACTCGATAT-3´.

The regulator and effector constructs were independently transformed into WT *Arabidopsis* via the *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998). Established effector lines for *CCA1*, *LHY*, and *TOC1* were crossed with plants carrying the regulator construct to yield *Alc::CCA1, Alc::LHY* and *Alc::TOC1.*

CAB2::LUC circadian reporter. A translation enhancer sequence, Ω (Gallie et al., 1987), was created by annealing the following complementary DNA oligos: *Omega*(+), 5´-AGCTTATTTTTACAACAATTACCAACAACAACAAACAACAAACAACATTA-

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CAATTACTATTTACAATTAC-3´ and *Omega*(–), 5´-CATGGTAATTGTAAATAGTAATTGTAAT-GTTGTTTGTTGTTTGTTGTTGTTGGTAATTGTTGTAAAAATA-3´. Double-stranded Ω was inserted into the *Xho*I/*Hin*dIII sites of pGL3-Promoter Vector (Promega Corp., Madison, WI), yielding an Ω*:LUC* fusion. Ω*:LUC* was then subcloned into the *Bam*HI/*Xba*I sites of the plant binary vector pKYLX71 (Schardl et al., 1987) (the 35S promoter within pKYLX71 had been previously removed by *Eco*RI + *Xho*I digestion). The resulting promoter-less vector was called pKYLX-Ω*:LUC.* To create the *CAB2::LUC* reporter construct, the –222 to +7 region (relative to transcription start) of the *CAB2* promoter was amplified from *Arabidopsis* gDNA by PCR using the primers *CAB2p*_F, 5´-GGTACCGTTGAAG-TATTCAG-3´, and *CAB2p*_R, 5´-CTCGAGAGTGAT-TAAAACTG-3´. The amplified *CAB2* promoter fragment was ligated upstream of Ω in pKYLX-Ω*:LUC* at the *Sac*I/*Xho*I sites, yielding pKYLX-*CAB2::LUC. CAB2::LUC* was transformed into WT, *Alc::CCA1*, *Alc::TOC1*, and *Alc::LHY* plants.

RNA quantitation. Total RNA was extracted from 8-day-old seedlings as described (Chang et al., 1993). The RNA was treated with RQ1 DNase (Promega Corp., Madison, WI) and reverse transcribed using random hexamer primers and M-MLV Reverse Transcriptase (Invitrogen Corp., Carlsbad, CA) following the manufacturer's instructions. Quantitative RT-PCR reactions were carried out on an MX3000P machine (Stratagene, La Jolla, CA) using general PCR reagents (Denville Scientific, Metuchen, NJ) supplemented with 0.5X SYBR Green I (diluted from a "10,000 X" stock; Invitrogen Corp.). *RNA HELICASE 8* (*RH8*; Schaffer et al., 2001) was used as a noncycling control. Reactions were performed in triplicate.

Quantitative RT-PCR primers

CCA1_F 5´-GGGGTGTGAATGATGGAAAAGA-3´
CCA1_R 5´-CGATCTTCATTGGCCATCTCAG-3´
CCR2_F 5´-GCTCTTGAGACTGCCTTCGCTC-3´
CCR2_R 5´-CTCGTTAACAGTGATGCTACGG-3´
GI_F 5´-AAGCGACTTTTAGACGATGTGCTG-3´
GI_R 5´-GATTGGAAGAATGACTGCATGACC-3´
LHY_F 5´-GACAACGCGGTTCAAGATGTTC-3´
LHY_R 5´-CCAAGGGTAGTTTTGCATGCTG-3´
LUX_F 5´-AGCCGTACGATTCAGGATGTGA-3´
LUX_R 5´-CTCGATCTTCCTCCTCCACGTT-3´
PRR9_F 5´-ATTGACAAGCGTCCTGATAGTATTTAT-3´
PRR9_R 5´-AACTTACAGAGCAAGATCTTTTCAGAG-3´
RH8_F 5´-CAATGGCTTCGAAGAGGTCAGA-3´
RH8_R 5´-TGGGTCGATAACTTCGGATTGA-3´
TOC1_F 5´-GTGGTCTTGGTGCTGATGGAAC-3´
TOC1_R 5´-GACCCAGCAAGATGATCCAATG-3´

Anti-TOC1 antibody production. A fragment of the *TOC1* open reading frame encoding amino acid residues 164 to 294 was amplified by PCR using the primers 5´-GGGATCTGAATTCAGTGATCCAAA-CAC-3´ and 5´-AAGATGACTCGAGCCATTTC-CATCGAC-3´. The fragment was introduced into the *Eco*RI/*Xho*I sites of the vector pGEX-4T-1 (Pharmacia, Uppsala, Sweden). Recombinant GST-TOC1(164-294) was expressed in *E. coli* BL21/DE3 cells and purified using glutathione sepharose (GE Healthcare, Buckinghamshire, UK) as described (Wang et al., 1997). Recombinant proteins were used to generate polyclonal anti-TOC1 antiserum (Harlan Bioproducts for Science, Inc., Indianapolis, IN). Antiserum was subjected to immunoaffinity purification against GST-TOC1-fragment immobilized to a nitrocellulose membrane as described (Olmsted, 1981).

Western blotting. Protein from 8-day-old seedlings was extracted in 1× urea/SDS buffer (4 M urea, 2.5% SDS, 20 mM Tris-HCl, pH 6.8, and 0.05 mM EDTA, pH 8.0) containing 1× protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN), 2 mM PMSF, 50 M MG115, and 50 M MG132. Protein concentrations were measured with a DC protein assay reagent (Bio-Rad, Hercules, CA) and 50 g of total proteins was separated on 8% (w/v) SDS-polyacrylamide gels. Proteins were electrophoretically transferred to PVDF membranes (Millipore, Bedford, MA). Immunoblotting procedures were performed as described (Lu and Hrabak, 2002) using antibodies to CCA1 (Wang and Tobin, 1998), LHY (Daniel et al., 2004), TOC1, or actin. Quantitation of immunodetected proteins was performed using ImageJ software (http://rsb.info.nih.gov/ij/).

EtOH pulse. Transient activation of the EtOH-inducible system was achieved by short exposures (≤ 20 min) of 8- to 11-day-old seedlings to EtOH vapor. The tissue culture plates served as convenient vapor chambers. For seedlings on 90-mm plates, inductions were carried out by wetting a paper filter (83-mm diameter; Chromatography Paper (thick);

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Fisher Scientific Inc., Hampton, NH) with diluted EtOH (in water) and adhering it to the underside of the plate lid. For seedlings on 24-well plates, paper filters of dimensions 10.7 × 13.5 cm were adhered to the inner lid of a P5000 pipette tip box (Denville Scientific, Metuchen, NJ). The concentration of the EtOH was 1% (v/v) unless stated otherwise. The seedlings were moved to fresh MS1.5 after treatment to avoid continuous induction from residual EtOH.

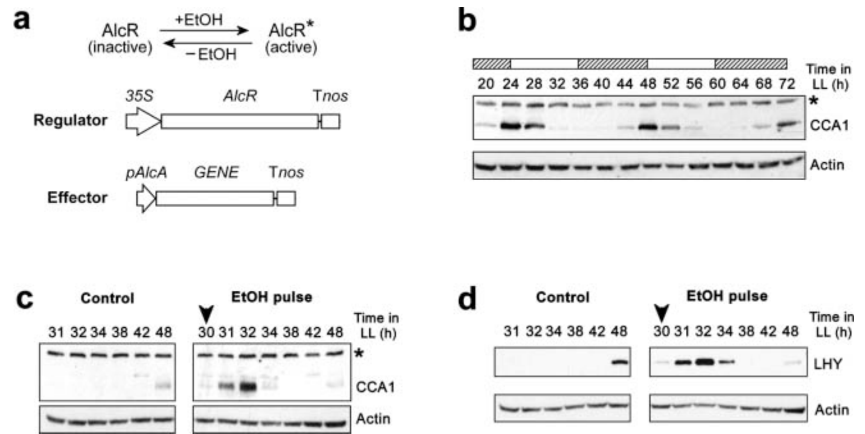

Figure 1. Ethanol (EtOH)-mediated pulses of *CCA1* and *LHY* expression in plants. (a) DNA constructs for the EtOH-inducible system. The ability of AlcR to function as a transcriptional activator is dependent on the presence of EtOH. Active AlcR binds the inducible promoter, *pAlcA*, and promotes transcription of the gene-of-interest. T_{nos} = terminator of the *NOS* gene; *35S* = constitutive promoter. (b) Western blot showing the rhythm of CCA1 abundance in WT plants under continuous light (LL). (c, d) Western blots showing pulses of protein following an EtOH vapor treatment at 30 h in LL. (c) Pulse of CCA1 protein in *Alc::CCA1* seedlings. (d) Pulse of LHY protein in *Alc::LHY* seedlings. Treatment time is marked by the arrowhead. Control plants were not treated. Asterisk = nonspecific band. Subjective day and subjective night are denoted by white and hatched bars, respectively.

Bioluminescence assays. Groups of approximately 20 *CAB2::LUC* seedlings were grown on 15-mm-diameter nylon net filters (20 mm pore size; Millipore Corp., Bedford, MA) on MS1.5 for 6 days in 12:12. The seedlings were then transferred to wells of a 24-well tissue culture plate (#3047 Falcon; Becton Dickinson & Co., Franklin Lakes, NJ) that contained 0.6 mL of MS1.5 and 1 mM D-luciferin (catalog number L-8220; Biosynth AG, Switzerland). The seedlings were incubated in 12:12 for 2 more days before transfer to continuous light (LL) for the experiment.

Six-minute exposures were acquired with a CCD camera (Princeton Instruments VersArray 512B; Roper Scientific, Inc., Trenton, NJ) every 60 min. The LL light source was a mixture of red (λ_{max} = 660 nm) and blue (λ_{max} = 470 nm) light-emitting diodes with a combined fluence rate of approximately 30 mol · m^{-2} · sec^{-1}. Luciferase activity was quantified using ImageJ software. Rhythmic period was measured using the freeware program PEANUTS (http://www.vu-wien.ac.at/i128/Peanuts.htm) that calculates Lomb-Scargle periodograms.

Phase shift measurements. Groups of T2 *CAB2::LUC* seedlings in homozygous WT, *Alc::CCA1*, *Alc::LHY*, and *Alc::TOC1* backgrounds were treated with EtOH vapor at different times during the 4th cycle in LL (72 to 96 h). Rhythmic luciferase activity was subsequently monitored for 4 days. Relative phase shifts in *CAB2::LUC* rhythms between control (untreated) and EtOH-treated groups were measured by cross-correlation analysis (Levine et al., 2002). Data from the first 24 h after treatment were not included in the analysis due to a temporary increase in *CAB2::LUC* activity caused by EtOH directly and/or movement of seedlings to fresh medium. Phase shifts and times of EtOH treatment were converted to circadian time (CT) by multiplying by (24/free running period (h)). Average free running period ± SEM of *CAB2::LUC* rhythms in various backgrounds: WT, 25.84 ± 0.14 h (n = 8); *Alc::CCA1*, 25.44 ± 0.13 h (n = 15); *Alc::LHY*, 25.58 ± 0.29 h (n = 6); and *Alc::TOC1*, 25.72 ± 0.21 h (n = 24).

RESULTS AND DISCUSSION

To give a pulse of putative oscillator components, we utilized the EtOH-inducible system from *Aspergillus nidulans* (Caddick et al., 1998; Roslan et al., 2001). The reasons for choosing this system were three-fold. First, the system showed promise for being capable of generating short pulses (<24 h) of gene expression (Deveaux et al., 2003). Second, it is a relatively simple system as it comprises just two expression constructs (Fig. 1a). Third, it was unlikely that the inducer would affect the circadian clock

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mechanism because very low concentrations of EtOH are sufficient for induction (Roslan et al., 2001). The requirement that the inducer must not affect circadian rhythms ruled out the option of using a heat-shock inducible system because the *Arabidopsis* circadian clock can use temperature changes as input for entrainment (Somers et al., 1998).

CCA1, *LHY*, and *TOC1* were used as effectors (inducible genes; Fig. 1a) and introduced into plants transformed with the regulator construct. The resulting plant lines were called *Alc::CCA1*, *Alc::LHY*, and *Alc::TOC1*. The circadian oscillations of endogenous CCA1 and LHY protein show peaks of expression at subjective dawn (Fig. 1b, and Daniel et al., 2004). EtOH treatment of *Alc::CCA1* or *Alc::LHY* seedlings in the middle of the subjective day, when endogenous CCA1 and LHY levels are low, resulted in a substantial increase in their RNA (Fig. S1a, b; Figures S1-S6 available online at http://jbr.sagepub.com/supplemental/) and protein (Fig. 1c, d). EtOH-induced *CCA1* RNA peaked 1 h after treatment and then rapidly declined. The peak level of induced *CCA1* exceeded the peak level of endogenous *CCA1* RNA by approximately four-fold (Fig. S1a). Induced CCA1 protein peaked 2 h after treatment and also exceeded its endogenous peak level at 48 h in LL (Fig. 1c). Similar results were obtained for EtOH treatment on *Alc::LHY* plants, except that the peak level of induced *LHY* was less than two-fold higher than the peak level of endogenous *LHY* RNA (Fig. S1b). By treating with EtOH at various times in LL, we found that induction of either protein can be carried out at any point in the circadian cycle (Fig. S2).

To monitor circadian rhythms over time we utilized the *CAB2::LUC* circadian reporter (Millar et al., 1995), which was transformed into the plants containing the inducible *Alc* constructs. EtOH treatment given at four different times in the circadian cycle did not affect the luciferase rhythms observed in WT plants (Fig. 2a). However, when *CCA1* or *LHY* expression was induced by EtOH

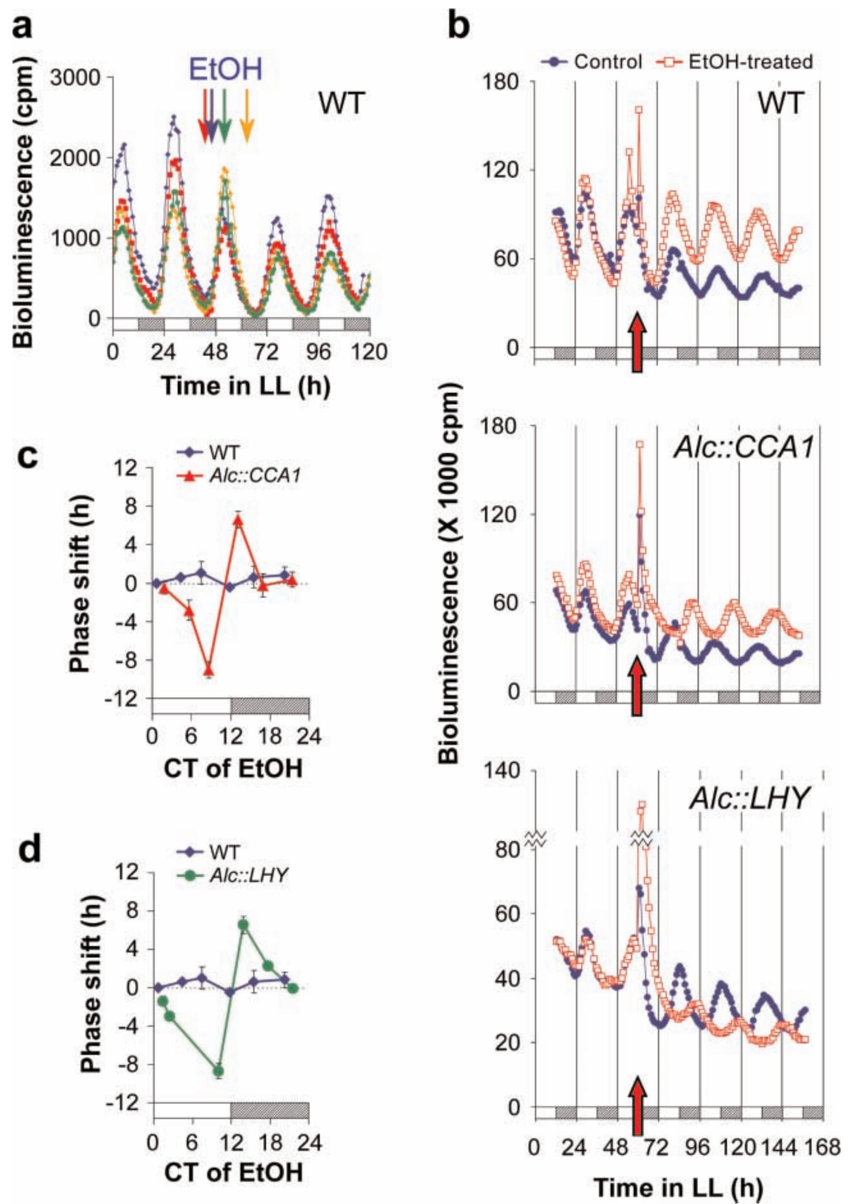

Figure 2. A pulse of *CCA1* or *LHY* expression is sufficient to reset the circadian clock. (a) Ethanol (EtOH) does not affect the circadian phase of *CAB2::LUC* rhythms in WT seedlings. Each plot represents the mean bioluminescence of 6 individual seedlings. Colored arrows represent times of a 20-min exposure to 10% (v/v) EtOH for their corresponding colored plots. (b) Effect of a 1% (v/v) EtOH pulse on the phase of *CAB2::LUC* rhythms in WT, *Alc::CCA1*, and *Alc::LHY* seedlings. The times of treatment with EtOH are denoted by a red arrow. Plots represent the mean bioluminescence of 20 seedlings. (c, d) Phase-response curves (PRCs) for a 1% (v/v) EtOH pulse on WT plants versus *Alc::CCA1* (c) and *Alc::LHY* (d). Seedlings were treated with EtOH at different times in LL and the resulting phase shifts in *CAB2::LUC* rhythms were plotted as a function of treatment time. Delays are negative phase shifts and advances are positive. Error bars denote ± SD ($n = 2$ to 9). CT = circadian time; cpm = counts per minute.

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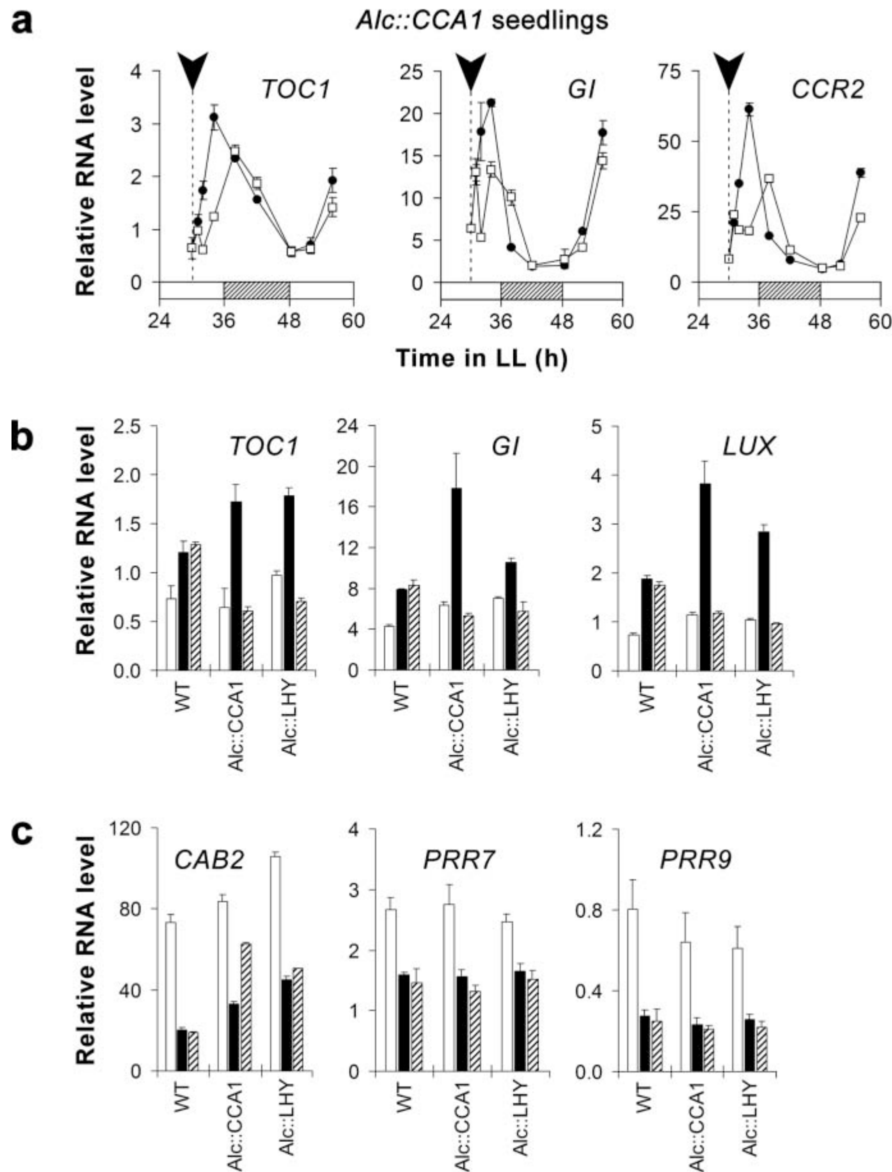

Figure 3. Effect of induced *CCA1* or *LHY* expression in the middle of the subjective day (CT 6) on the expression of clock-controlled genes. (a) A pulse of *CCA1* expression delays the peak of *TOC1*, *GI*, and *CCR2* RNA. *Alc::CCA1* seedlings were given an ethanol (EtOH) pulse at the time indicated by the arrowhead. Closed circles = untreated plants; open squares = EtOH-treated. (b) A pulse of *CCA1* or *LHY* represses the accumulation of *TOC1*, *GI*, and *LUX* RNA. (c) Effect of *CCA1* and *LHY* inductions on the accumulation of *CAB2*, *PRR7*, and *PRR9* RNA. WT, *Alc::CCA1*, and *Alc::LHY* seedlings were given an EtOH pulse at 30 h in LL (CT 6). White bar = relative RNA level at the time of induction; black bar = level after 2 h (no EtOH); hatched bar = level 2 h after EtOH. RNA abundance was measured by quantitative RT-PCR and normalized to *RNA HELICASE 8* (*RH8*). Error bars denote ± SD ($n = 3$).

at subjective dusk, the luciferase rhythm was shifted by 9 h (Fig. 2b). The phase shifts were evident within 24 h of the treatment and were remarkably stable as they did not revert back to the original entrained phase even after 13 days (Fig. S3). By treating with EtOH at various times we constructed phase-response curves (PRCs) for the CCA1 and LHY pulses (Fig. 2c, d). The PRCs showed that EtOH treatment near subjective dawn (circadian time 0 (CT 0)) did not cause phase shifts. This result was expected because CCA1 and LHY are already abundant at this point in the circadian cycle. Relatively large phase shifts were elicited when the EtOH treatment was given near CT 12, when endogenous CCA1 and LHY levels are low. In contrast, the PRC for WT plants showed no phase shifts for the EtOH treatments. These results demonstrate that a pulse of expression of either *CCA1* or *LHY* is sufficient to reset the phase of the circadian clock. The PRCs for a *CCA1* or *LHY* pulse resemble the published PRC for a red light pulse (Covington et al., 2001), with phase delays and advances occurring in the subjective day and subjective night, respectively. Because *CCA1* and *LHY* expression is induced by red light (Wang et al., 1997; Kim et al., 2003), this result is consistent with the hypothesis that light-induced changes in their expression contribute to the entrainment of the circadian clock by light. Interestingly, an analogous mechanism is present in the *Neurospora* clock, where resetting of the clock to light is mediated by *FREQUENCY*, a central oscillator gene whose expression is also induced by light (Crosthwaite et al., 1995).

To investigate whether the observed phase shift in *CAB2::LUC* rhythms is due to resetting of the central clock, the effect of a *CCA1* pulse on the rhythms of other clock-controlled genes was examined. When *CCA1* was induced during the subjective day (CT 6), the RNA accumulation of *TOC1*

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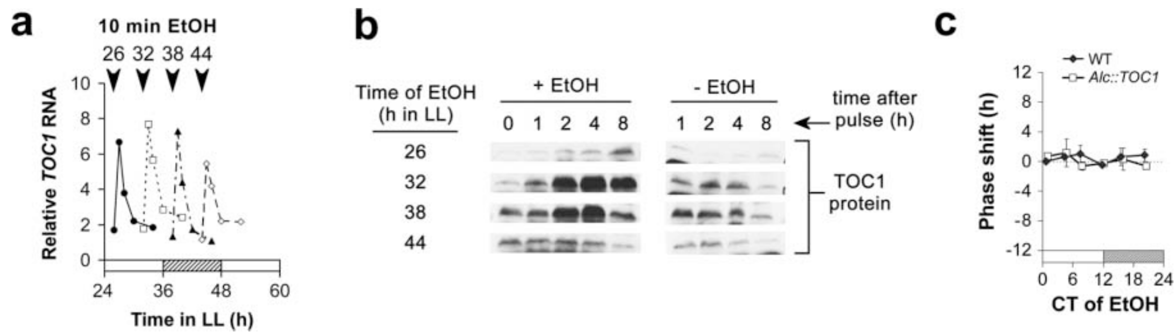

Figure 4. Induction of TOC1 protein in *Alc::TOC1* seedlings is restricted to the subjective evening. (a, b) *Alc::TOC1* seedlings were given a 3% ethanol (EtOH; v/v) pulse at various times (26, 32, 38, and 44 h in LL) and tissue was harvested over time. (a) *TOC1* RNA is EtOH inducible at any point in the circadian cycle in the *Alc::TOC1* line. RNA levels were measured by quantitative RT-PCR and were normalized to levels of *RH8* RNA. Arrowheads denote times of EtOH treatment for their respective plot. Induction times in LL: circles = 26 h; squares = 32 h; triangles = 38 h; diamonds = 44 h. (b) Western blots showing the accumulation of TOC1 protein following the EtOH pulses. Total protein extracts were isolated from the same tissue as used in (a) and were processed by SDS-PAGE. Blots were probed with purified TOC1 antibodies. (c) Phase-response curve for an EtOH pulse on WT versus *Alc::TOC1* seedlings. Error bars denote ± SD (*n* = 2 to 9).

and *GI* was delayed (Fig. 3a). A phase delay was also detected in the RNA accumulation of the evening-phased output gene *COLD-CIRCADIAN RHYTHM-RNA BINDING 2* (*CCR2*; Carpenter et al., 1994; Fig. 3a). Additionally, peaks of *TOC1* and *CCR2* RNA were shifted by 4 h, which is consistent with the observed shift in *CAB2::LUC* rhythms when *CCA1* expression was induced at a similar time (compare Fig. 3a with Fig. 2c). A pulse of *LHY* expression at CT 6 resulted in a similar phase delay in *TOC1* expression (Fig. S4). Together, these results demonstrate that a pulse of expression of either *CCA1* or *LHY* can set the phase of the circadian clock.

It has been suggested that CCA1 and LHY function as negative elements of the central oscillator to inhibit transcription of positive elements (Alabadí et al., 2001). Therefore, we examined the effect of a pulse of *CCA1* or *LHY* on the expression of proposed central oscillator genes *TOC1*, *GI*, and *LUX*. As shown in Figure 3b, a pulse of either *CCA1* or *LHY* at a time when *TOC1*, *GI*, and *LUX* RNA levels are increasing (CT 6) caused a virtually complete inhibition of the increase within 2 h of the EtOH treatment. This result is consistent with *CCA1* and *LHY* functioning as transcriptional repressors in the central oscillator. In addition to their roles as repressors, CCA1 and LHY have been proposed to activate expression of *CAB2*, *PRR7*, and *PRR9* (Wang et al., 1997; Farré et al., 2005). Figure 3c shows that a *CCA1* or *LHY* pulse at CT 6 inhibited the decline of *CAB2* RNA. The fact that the inhibition caused by the *LHY* pulse was less than that for *CCA1* is likely to be because of the weaker induction of *LHY* relative to its circadian peak level (Fig. 1c, d; Fig. S1a, b). However, neither *PRR7* nor *PRR9* expression was affected by the pulses (Fig. 3c); thus neither CCA1 nor LHY alone are sufficient to activate their expression. Together these results support the idea that CCA1 and LHY are involved in both the activation and the repression of clock-associated genes.

In the core feedback loop of the central oscillator, TOC1 has been proposed to act as an indirect activator of *CCA1* and *LHY* expression (Alabadí et al., 2001). To determine whether a pulse of *TOC1* can also reset the phase of the circadian clock, *Alc::TOC1* plants were generated. An EtOH treatment is able to induce *TOC1* RNA to approximately 4-fold the level of its endogenous peak, which is comparable to *CCA1* induction in *Alc::CCA1* plants (Fig. S1a, c). To confirm that the RNA induction leads to accumulation of TOC1 protein, TOC1-antibodies were generated (Fig. S5). While *TOC1* RNA was induced to a similar level at any point in the circadian cycle (Fig. 4a), TOC1 protein was not (Fig. 4b). When EtOH treatment was given near subjective dusk (32 and 38 h in LL), at a time of high endogenous *TOC1* expression, TOC1 protein peaked 2 to 4 h after treatment and exceeded the peak level of the endogenous protein (Fig. 4b). However, only a weak accumulation of TOC1 was detected when the inductions were performed near subjective dawn (26 and 44 h in LL),

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antiphasic to its normal circadian peak (Fig. 4b). These results indicate that the synthesis and/or stability of TOC1 protein is temporally regulated, perhaps by the circadian clock itself. TOC1 protein stability is known to be modulated by ZEITLUPE (ZTL), an F-box protein that also functions as a circadian photoreceptor (Más et al., 2003; Kim et al., 2007). ZTL interacts with TOC1 and targets it for degradation through the 26S proteasome pathway (Más et al., 2003). PRR3, whose expression also peaks in the evening, was recently proposed to function as an inhibitor of this interaction, thereby making TOC1 more stable (Para et al., 2007). It is possible that the induced TOC1 protein in *Alc::TOC1* seedlings at subjective dawn is degraded rapidly through its interaction with ZTL, perhaps due to the lack of protection from PRR3.

To examine whether the weak induction of TOC1 protein near subjective dawn and the increased peak level near subjective dusk in *Alc::TOC1* plants affect the phase of the circadian clock, we generated a PRC, again using *CAB2:: LUC* as a circadian reporter. We could not detect any phase shifts in rhythms following *TOC1* induction at different CTs (Fig. 4c). To rule out the possibility that the induced TOC1 protein is inactive, we induced its expression continuously and measured the period length of the *CAB2::LUC* rhythm. Increased TOC1 dosage has been shown to result in long-period rhythms (Más et al., 2003). Figure S6, in the supplementary information, shows that constant induction of *TOC1* expression did result in a period lengthened by 6 h, demonstrating that the induced TOC1 is functional. This finding is in agreement with previous reports that found that TOC1 protein level is significantly affected in both *prr3* and *ztl* mutants (Más et al., 2003; Para et al., 2007), suggesting that TOC1 is important for the regulation of circadian period by the clock.

In this work, we have demonstrated that a pulse of *CCA1* or *LHY* expression in plants is sufficient to reset the circadian clock, showing that both proteins function as phase-setting components of the *Arabidopsis* circadian clock and fulfill one of the proposed criteria for central oscillator components. In addition, the observation that the induced TOC1 protein accumulates only during the subjective evening suggests that *TOC1* expression is tightly regulated by the circadian clock and that its protein must exceed a certain level for it to perform its clock-associated function(s). Moreover, we show that the use of the EtOH-inducible system can provide a means for experimentally testing the effect of a pulse of virtually any gene on a biological pathway.

ACKNOWLEDGMENTS

We thank Jackie Paine at Zeneca for providing us with the initial plasmid and guidelines for using the EtOH-inducible system, Dr. Steve Kay's group for providing us with *toc1-4* seeds, and Dr. Takeshi Mizuno for providing PRR1-OX seeds. We also thank Drs. Chentao Lin and Candace Webb for thoughtful suggestions and critical reading of this manuscript, and Carole Gorin for her excellent administrative assistance. This work was supported by NIH grant GM23167 to E.M.T. and the George Laties Fellowship to S.M.K.

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