

# Positive and Negative Factors Confer Phase-Specific Circadian Regulation of Transcription in Arabidopsis <sup>W</sup>

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**The circadian clock exerts a major influence on transcriptional regulation in plants and other organisms. We have previously identified a motif called the evening element (EE) that is overrepresented in the promoters of evening-phased genes. Here, we demonstrate that multimerized EEs are necessary and sufficient to confer evening-phased circadian regulation. Although flanking sequences are not required for EE function, they can modulate EE activity. One flanking sequence, taken from the *PSEUDO RESPONSE REGULATOR 9* promoter, itself confers dawn-phased rhythms and has allowed us to define a new clock promoter motif (the morning element [ME]). Scanning mutagenesis reveals that both activators and repressors of gene expression act through the ME and EE. Although our experiments confirm that CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) are likely to act as repressors via the EE, they also show that they have an unexpected positive effect on EE-mediated gene expression as well. We have identified a clock-regulated activity in plant extracts that binds specifically to the EE and has a phase consistent with it being an activator of expression through the EE. This activity is reduced in *CCA1/LHY* null plants, suggesting it may itself be part of a circadian feedback loop and perhaps explaining the reduction in EE activity in these double mutant plants.**

## INTRODUCTION

Circadian rhythms are pervasive throughout nature. These self-sustaining rhythms are thought to provide an adaptive advantage by allowing organisms to anticipate the regular changes in the environment that occur as a result of the earth's rotation on its axis (Ouyang et al., 1998; Green et al., 2002; Michael et al., 2003). A wide variety of processes are influenced by the circadian clock in plants, including the transition from vegetative to reproductive growth, photosynthetic capacity, and regulation of gene expression. Circadian regulation of gene expression has been observed in all organisms with a functional clock and is thought to play an important role in the central oscillator in many organisms (Harmer et al., 2001), although the core oscillator in cyanobacteria may run independently of transcription (Tomita et al., 2005). Genome-wide studies have shown that a sizable portion of the transcriptome is under circadian regulation in plants, cyanobacteria, animals, and fungi (reviewed in Duffield, 2003; Sato et al., 2003). Microarray and enhancer trapping experiments have led to estimates that between 2 and 36% of the *Arabidopsis thaliana* genome is under circadian transcriptional regulation (Harmer et al., 2000; Schaffer et al., 2001; Michael and McClung, 2003). These transcripts show peak abundance at all phases of the

circadian day and night, suggesting that a complex network of transcription factors is used to generate this variety of phases.

In theory, a circadian oscillator could be based upon a single feedback loop. However, in practice it seems that clocks are generally composed of multiple interlocked feedback loops, perhaps to provide robustness of rhythms (Roenneberg and Merrow, 2003; Emery and Reppert, 2004). A model describing the central feedback loop in Arabidopsis has been proposed (Alabadi et al., 2001). The homologous Myb-like transcription factors CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) are thought to act around dawn to repress the expression of the pseudoreponse regulator *TIMING OF CAB EXPRESSION 1 (TOC1)* (also known as Arabidopsis *PSEUDO RESPONSE REGULATOR 1 [PRR1]*) by binding to a motif in the *TOC1* promoter termed the evening element (EE). *TOC1* levels increase toward the end of the day and are thought to directly or indirectly increase expression of *CCA1* and *LHY*. Although this model is consistent with much of the published data, it does not explain the roles of the evening-phased genes *GIGANTEA (GI)*, *EARLY FLOWERING 3 (ELF3)*, or *ELF4*, all of which are required for the expression of *CCA1* and *LHY* (Schaffer et al., 1998; Park et al., 1999; Doyle et al., 2002). In addition, there are four clock-regulated *TOC1* homologs (*PRR3*, *PRR5*, *PRR7*, and *PRR9*) that show peak expression at various times and that appear to act close to the circadian oscillator (Farre et al., 2005; Nakamichi et al., 2005a, 2005b; Salome and McClung, 2005). Available data, although insufficient to derive a detailed model, suggest that these factors act together in complex interdependent feedback loops to control rhythms in Arabidopsis (for review, see Salome and McClung, 2004).

One approach to understanding the circadian clock is to study the regulation of clock output genes and then work backward

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toward the central oscillator. In a previous study, we used such an approach to identify the EE (Harmer et al., 2000). We found that this nine-nucleotide sequence (AAAATATCT) was overrepresented in the promoters of clock-regulated genes, most of which had evening-phased peak expression. The EE has been shown to be important for the rhythmic activity of several evening-phased promoters: portions of promoters as small as 130 bp containing one EE confer evening-phased rhythms on a reporter gene, and this rhythmicity is abrogated by mutation of the EE (Harmer et al., 2000; Alabadi et al., 2001; Michael and McClung, 2002). A highly related motif, the CCA1 binding site (CBS; AAAAATCT) is found in the promoters of many day-phased genes (Wang et al., 1997; Piechulla et al., 1998; Michael and McClung, 2003). It has been suggested that the CBS suffices to confer dawn-phased rhythmic expression mediated by the positive actions of CCA1 and LHY (Michael and McClung, 2002). Other motifs have been reported to be overrepresented in the promoters of clock-regulated genes in plants, but the functional significance of these findings has not yet been reported (Hudson and Quail, 2003; Michael and McClung, 2003).

In this study, we further investigate the role of the EE in circadian regulation of gene expression. We have found that the EE itself, when multimerized, is necessary and sufficient to confer evening-phased rhythms on a reporter gene. This regulation does not require sequences normally found flanking the EE in clock-regulated promoters. Using genetic and biochemical tools, we provide data confirming that CCA1 and LHY act as repressors through minimal EE-containing enhancer elements. However, genetic data suggest that these factors play an additional positive role in EE-mediated gene expression. Although the EE is sufficient to confer rhythms, our data suggest that flanking regions can modulate the function of the EE, affecting rhythmicity, phase, amplitude, and expression level of the reporter gene. One region flanking the EE in the *PRR9* promoter is itself sufficient to confer dawn-phased rhythms on a reporter gene, making it a circadian morning element (ME). Scanning mutagenesis of the ME and EE demonstrates that both positive and negative regulators of transcription bind to these motifs, and both types of factors are required for robust rhythms. In an effort to identify *trans*-acting factors, we have found a clock-regulated EE binding activity in plant extracts that may correspond to the EE-activating factor. The abundance of this activity is dependent upon *CCA1* and *LHY*, perhaps explaining the reduction of EE activity in *CCA1/LHY* double mutant plants. This unknown factor may therefore act in a secondary feedback loop that helps regulate expression of *TOC1* and other evening-phased genes.

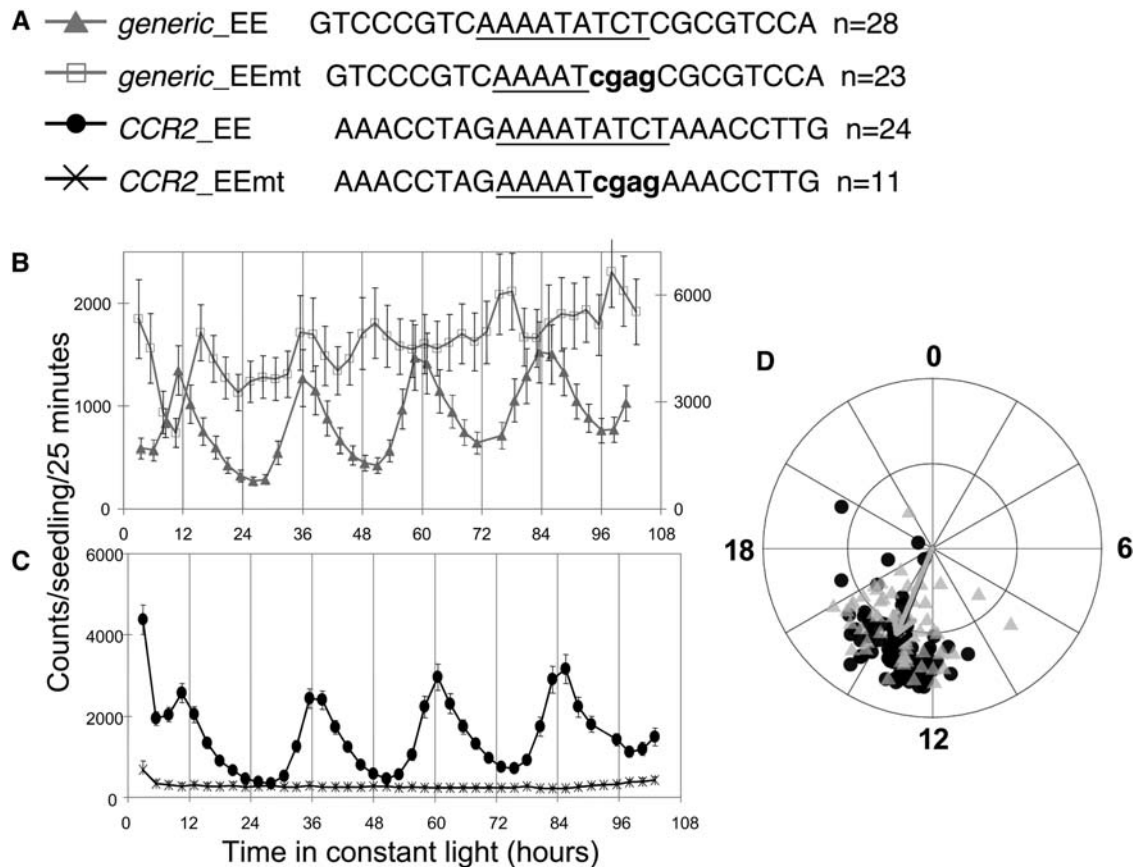
## RESULTS

### The EE Is Necessary and Sufficient to Confer Evening-Phased Gene Expression

To better understand the role of the EE in the regulation of clock-controlled genes in Arabidopsis, we used luciferase reporter constructs to determine whether the EE alone is sufficient to confer rhythmic transcription. Rather than studying a limited number of T2 families, we chose to examine luciferase expres-

sion in a large number of T1 plants to help control for differences in gene expression due simply to transgene insertion site. Four tandem repeats of the EE separated by 16 randomly selected nucleotides were placed upstream of the minimal promoter region of the nopaline synthase (NOS) gene and the modified firefly luciferase coding region. Half of the drug-resistant T1 plants transformed with this construct (*generic\_EE*) exhibited detectable luciferase activity. Of the visible T1 transformants, 92% exhibited circadian rhythms in luciferase activity, with the average peak phase of luciferase activity at circadian time 13.5 (CT 13.5, or 13.5 h after subjective dawn) (Figure 1, Table 1). To verify the importance of the EE for conferring evening-phased rhythms, we mutated the last 4 bp of each EE and transformed plants with this construct (*generic\_EEemt*). Only 9% of visible plants transformed with this construct exhibited circadian rhythms in luciferase activity (Figure 1, Table 1). Because it has been variously estimated that between 2 and 36% of the Arabidopsis transcriptome is clock regulated (Harmer et al., 2000; Schaffer et al., 2001; Michael and McClung, 2003), this cycling is likely due to insertion of the *generic\_EEemt* construct into genome regions regulated by the promoters of endogenously cycling genes. Average luciferase activity was significantly greater in lines generated with this construct when compared with those generated with the wild-type EE construct (Table 1), suggesting that mutation of the EE disrupted the binding site of an inhibitor of transcription. Plants were also transformed with a construct containing only the NOS minimal promoter driving luciferase; very few had visible luciferase activity, and these rare visible plants did not show clock regulation of bioluminescence (data not shown). These data demonstrate that the EE is necessary and sufficient to confer evening-phased rhythms.

We next wanted to determine whether sequences flanking the EE in endogenous promoters could modify EE activity. We therefore created a vector with a synthetic enhancer based upon an EE-containing region in the promoter of an evening-phased gene, *COLD AND CLOCK REGULATED 2* (*CCR2*; also known as *GLYCINE-RICH RNA binding PROTEIN 7*). We placed four direct repeats of 25 bp from this promoter (centered on an EE) upstream of the NOS minimal promoter (*CCR2\_EE*). Almost all plants transformed with this construct displayed circadian regulation of the reporter gene: 99% of detectably luminescent plants showed rhythmic luciferase activity (Figure 1, Table 1). Their average peak phase of expression was CT 13.4, very similar to the phase of 13.5 observed in the *generic\_EE* plants. Plants transformed with a construct in which the last 4 bp of each EE were mutated (*CCR2\_EEemt*) showed greatly reduced rhythmicity (36%;  $n = 5$ ) (Figure 1, Table 1). These few rhythmic plants did not display a consolidated phase of peak expression as seen for plants harboring either the *generic\_EE* or the *CCR2\_EE* constructs (data not shown). Interestingly, plants transformed with the *CCR2\_EEemt* construct exhibited a significantly lower average level of luciferase activity than plants transformed with the *CCR2\_EE* construct (Table 1) (in contrast with the higher expression seen in *generic\_EEemt* plants relative to *generic\_EE* plants). Thus, that mutation of the EE in the context of flanking *CCR2*-derived sequences disrupted the binding site of an activator of transcription.



**Figure 1.** The EE Is Necessary and Sufficient for Evening-Phased Rhythms.

**(A)** Reporter constructs were made with four copies of either wild-type or mutant EE sequences (shown) placed upstream of the NOS minimal promoter and modified firefly luciferase.

**(B)** *generic\_EE* and *generic\_EE*mt T1 transformants were grown on selective medium in 12-h-light/12-h-dark cycles for 8 d and then transferred to constant white light. Luminescence levels were monitored every 2.5 h for 5 d. Period, phase, and relative amplitude error (RAE; a measure of the robustness of the rhythm) were calculated according to the method of Plautz et al. (1997). The average luminescence ( $\pm$ SE of the mean) levels for the numbers of T1 plants noted in **(A)** are plotted. Data on additional T1 plants are presented in Table 1. *generic\_EE* plants are plotted on the primary and *generic\_EE*mt plants are plotted on the secondary y axis.

**(C)** *CCR2\_EE* and *CCR2\_EE*mt T1 plants were imaged and analyzed as described in **(B)**.

**(D)** Circular plot of the phase and RAE for all *generic\_EE* ( $n = 56$ ) and *CCR2\_EE* ( $n = 69$ ) T1 plants that returned a rhythm. Phase is indicated in CT (phase/period  $\times$  24 h) and RAE is graphed radially such that plants with RAE = 1 (no significant rhythm detected; error in the amplitude equals the amplitude value itself) would be graphed at the center of the circle and plants with RAE = 0 (very robust rhythms with an infinitely well determined rhythmic component) would be graphed on the periphery of the outermost circle.

### Sequences That Confer Dawn-Phased Cycling Overlap with the EE in the *PRR9* Promoter

Although most clock-regulated genes with an EE in their promoter regions have peak expression in the evening, there are a few exceptions. One is the pseudoresponse regulator *PRR9*, which has recently been shown to play an important role in regulation of the central circadian oscillator (Eriksson et al., 2003; Farre et al., 2005; Nakamichi et al., 2005a; Salome and McClung, 2005). *PRR9* expression peaks  $\sim$ 4 h after subjective dawn (CT 4) when plants are grown in constant light (Matsushika et al., 2000). In addition to an EE, this promoter also contains a CBS and two G-box motifs, which have also been implicated in circadian

regulation (Michael and McClung, 2002, 2003; Hudson and Quail, 2003). To determine the role of the EE in the context of the *PRR9* promoter, we created a synthetic enhancer with four direct repeats of a 25-bp fragment of the *PRR9* promoter inserted upstream of the NOS minimal promoter and luciferase. This construct is identical to the *generic\_EE* and *CCR2\_EE* constructs except for the *PRR9*-specific sequences flanking the EE. Again, this construct conferred rhythmic luciferase activity on most plants (95% of visibly bioluminescent plants showed circadian rhythms in luciferase activity). The average peak phase of expression occurred at CT 12.8, similar to the average phase of peak expression seen in *generic\_EE* and *CCR2\_EE* plants (Figures 2A and 2C, Table 1). This demonstrates that when

**Table 1.** Summary of T1 Plant Expression, Rhythmicity, and Phase Information

Construct	Independent Transgenics			Expression <sup>a</sup>			Rhythmic Plants <sup>b</sup>		Phase (CT) <sup>c</sup>	
	<i>n</i>	Visible	%	Average Counts	SE	P Value <sup>d</sup>	<i>n</i>	%	Average	SD
<i>generic_EE</i>	119	61	51.3	335.0	61.9	–	56	91.8	13.5	0.44
<i>generic_EE</i> mt	34	23	67.6	3069.2	626.5	2.8e-5	2	8.7	N.D.	N.D.
<i>CCR2_EE</i>	84	70	83.3	745.9	204.8	–	69	98.6	13.4	0.31
<i>CCR2_EE</i> mt	44	14	31.8	82.8	22.3	7.0e-8	5	35.7	N.D.	N.D.
<i>PRR9_EE</i>	71	64	90.1	185.3	27.7	–	61	95.3	12.8	0.51
<i>PRR9_EE</i> mt	43	24	55.8	138.4	77.1	N.D.	21	87.5	1.6	0.57
<i>PRR9_EE</i> mt	43	24	55.8	138.4	77.1	–	21	87.5	1.6	0.57
ME_scan1	103	13	12.6	24.4	3.7	0.006	3	23.1	N.D.	N.D.
ME_scan2	33	6	18.2	23.2	7.3	8.6e-6	2	33.3	N.D.	N.D.
ME_scan3	34	2	5.9	17.1	4.3	6.8e-8	1	50.0	N.D.	N.D.
ME_scan4	41	39	95.1	973.0	122.2	1.2e-10	33	84.6	5.0	1.92
ME_scan5	91	77	84.6	133.5	12.4	N.D.	43	55.8	2.5	1.57
<i>PRR9_EE</i>	71	64	90.1	185.3	27.7	–	61	95.3	12.8	0.51
EE_scan1	82	73	89.0	418.3	87.3	0.3	72	98.6	13.4	0.30
EE_scan_2	99	73	73.7	89.7	12.8	1.5e-4	68	93.2	12.4	0.23
EE_scan_3	38	27	71.1	1960.3	555.6	0.005	4	14.8	N.D.	N.D.
EE_scan_4	41	39	95.1	1057.7	138.5	3.6e-10	18	46.2	14.2	1.88
EE_scan_5	139	27	19.4	33.6	11.6	1.3e-15	13	48.1	12.9	1.12
EE_scan_6	63	53	84.1	81.9	9.9	0.01	51	96.2	13.1	0.50
<i>generic_CBS</i>	65	12	18.5	1127.2 <sup>e</sup>	35.4	N.D.	3	25.0	N.D.	N.D.
<i>CCR2_CBS</i>	69	30	43.5	1032.3 <sup>e</sup>	111.5	N.D.	23	76.7	11.8	0.32
<i>PRR9_CBS</i>	58	31	53.4	1035.0 <sup>e</sup>	62.8	N.D.	22	71.0	12.7	0.53

Plants were grown and assayed as described in Figure 1 and rhythmic parameters calculated according to the methods of Plautz et al. (1997). N.D., not determined.

<sup>a</sup> Expression is defined as luciferase counts/seedling/25 min.

<sup>b</sup> A plant is defined as rhythmic if fast Fourier transform nonlinear least squares analysis returned an RAE <1.

<sup>c</sup> Phase is calculated in circadian time (= estimated phase/period × 24 h).

<sup>d</sup> P values for expression were calculated by comparing reference with its derivative constructs (i.e., comparing each mutant to the construct listed first within each group) using the Kruskal-Wallis test, with Bonferroni correction for multiple testing.

<sup>e</sup> These plants were assayed using a different CCD camera; thus, luciferase activity levels can't be directly compared with those observed with other constructs.

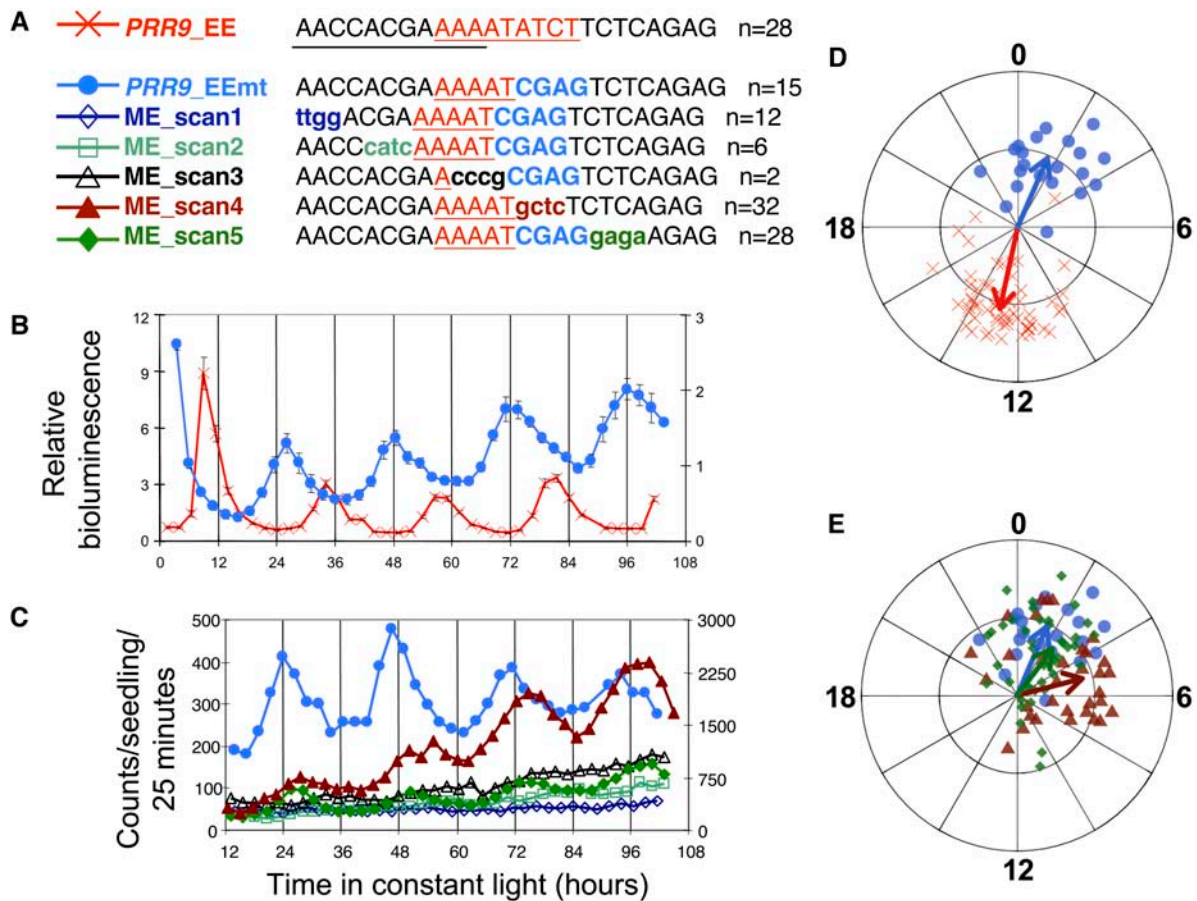
multimerized, the EE confers evening-phased rhythms even in the context of flanking sequences from a day-phased promoter.

To our surprise, we found that plants transformed with a mutant *PRR9* construct also demonstrated rhythmic luciferase activity. We altered the last four nucleotides of the EE to create *PRR9\_EE*mt, using the same base substitutions used to create the mostly arrhythmic *generic\_EE*mt and *CCR2\_EE*mt constructs. However, we found that the *PRR9\_EE*mt plants were largely rhythmic; almost all visible plants (88%) displayed circadian-regulated luciferase activity (Figures 2A and 2C, Table 1). Moreover, these rhythmic *PRR9\_EE*mt plants have peak luciferase activity shortly after subjective dawn, with an average phase of CT 1.6, almost 12 h out of phase with the *PRR9\_EE* plants. This demonstrates that the *PRR9\_EE*mt fragment contains an element sufficient to confer morning-phased rhythms (defined as the ME).

To better determine the nucleotides important for ME function, we performed scanning mutagenesis across the *PRR9\_EE*mt se-

quence, changing four nucleotides at a time (Figure 2E). Mutations within the 5' half of *PRR9\_EE*mt (ME\_scan1, ME\_scan2, and ME\_scan3) significantly reduced the luciferase activity of transformants (Table 1). Few drug-resistant plants transformed with these plasmids had detectable luciferase activity, and of these few were rhythmic. This suggests that these mutations disrupted the binding site of a transcriptional activator that may also be required for rhythm generation. Transgenic plants generated with a construct in which residues near the 3' end of this sequence were mutated (ME\_scan5) had average luciferase activity levels similar to those of *PRR9\_EE*mt plants but showed a reduced fraction of rhythmic seedlings (56% rather than 88%). The average time of peak expression of these ME\_scan5 plants is similar to that of *PRR9\_EE*mt plants (Table 1, Figures 2C and 2E). However, the phases of ME\_scan5 plants show greater variability than those of *PRR9\_EE*mt plants (Table 1, Figure 2E).

We next wished to determine whether ME activity is intrinsic to the *PRR9* promoter or whether our mutagenesis had created



**Figure 2.** The *PRR9* Promoter Contains MEs and EEs.

(A) Four copies of either the wild-type EE-containing region of the *PRR9* promoter or various mutants were placed upstream of luciferase, and T1 plants were assayed as described in Figure 1. The EE is indicated with red underlined text. The experimentally defined ME (this work) is underscored with a black line.

(B) Luciferase data for *PRR9\_EE* and *PRR9\_EE<sub>mt</sub>* plants. Data from each plant were normalized to its median expression level. Averages ( $\pm$  SE) of all plants that returned an RAE (i.e., for which a rhythm was detected) in this experiment are depicted, with *PRR9\_EE* plants plotted on the primary y axis and *PRR9\_EE<sub>mt</sub>* plants plotted on the secondary y axis.

(C) Average luminescence for T1 plants transformed with the mutant constructs indicated in (A). No RAE cutoff was applied, but only plants with detectable luciferase activity were considered. *EE\_scan4* plants are plotted on the secondary y axis, and all other lines are plotted on the primary y axis. The number of plants averaged for the data depicted in (B) and (C) is indicated in (A); data for additional T1 plants is summarized in Table 1.

(D) and (E) Phase and RAE are plotted for all *PRR9* and ME constructs that conferred substantially rhythmic luciferase activity (defined as >50% of the visible T1 plants transformed with a given construct showing statistically significant rhythms in luciferase activity).

a new motif. We therefore made another mutant, *ME\_scan4*. In this construct, we again mutated the last four nucleotides of the EE, but to different residues than those of *PRR9\_EE<sub>mt</sub>*. Of the luminescent *ME\_scan4:luc<sup>+</sup>* plants, 85% showed circadian rhythms of luciferase activity (Table 1, Figures 2C and 2E). Peak phases of luciferase activity were less consolidated than observed in the *PRR9\_EE<sub>mt</sub>* plants (Figure 2E), but their average phase of CT 5.0 is more similar to that observed in *PRR9\_EE<sub>mt</sub>* than in *PRR9\_EE* plants. Together, these data suggest that substantial ME function is contained within the 12 bp at the 5' end but that ME activity can be affected by downstream sequences. Consistent with this, *ME\_scan4* transformed plants

have significantly higher average luciferase activity than *PRR9\_EE<sub>mt</sub>* plants (Table 1).

### CBS Motifs Confer Evening-Phased Rhythms

Previous reports have suggested that the CBS, a motif that differs from the EE by only one nucleotide (AAAAAATCT versus AAAATATCT), confers dawn-phased rhythms on a reporter gene (Michael and McClung, 2002). These experiments were performed using a relatively large promoter fragment of 200 bp. To directly compare EE and CBS function in a minimal system, we

changed each EE within the three multimerized constructs described above to a CBS and generated transgenic plants. Few plants transformed with the *generic\_CBS* construct had detectable bioluminescence, and only one-quarter of these were clock regulated when assayed in constant light conditions (Table 1). By contrast, approximately half of the plants transformed with *CCR2\_CBS* or *PRR9\_CBS* constructs had visible luciferase activity, and most of these showed rhythmic luciferase activity in constant light. Surprisingly, these plants showed peak expression in the subjective evening, with average acrophase occurring at CT 11.8 for *CCR2\_CBS* and at CT 12.7 for *PRR9\_CBS* plants (Figure 3, Table 1). We observed the same evening phase of luciferase activity when these plants were assayed in constant darkness (data not shown). Thus, when multimerized, the CBS confers the same evening-phased rhythms as the EE.

### CCA1 and LHY Both Positively and Negatively Regulate EE-Mediated Gene Expression

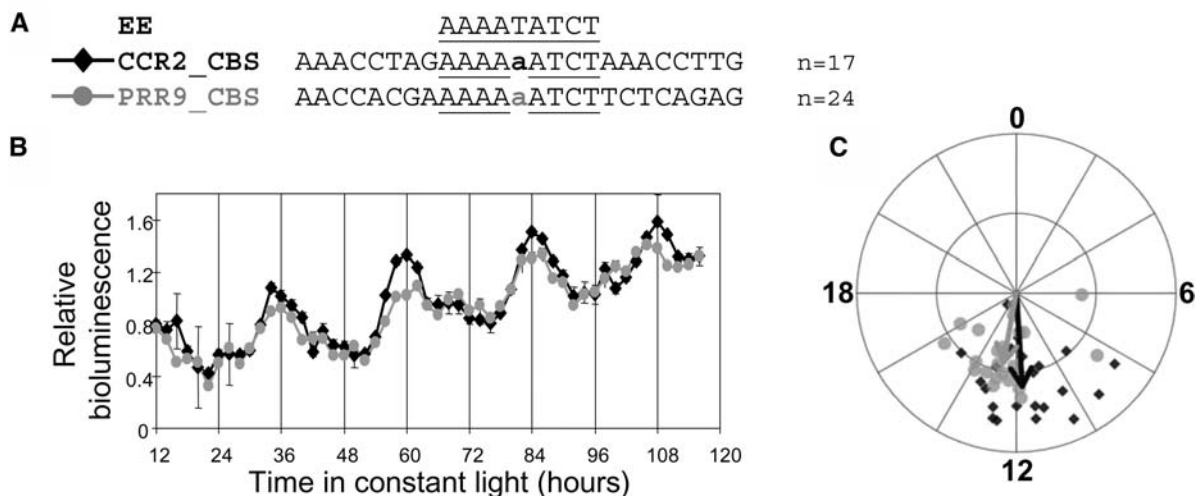
Previous work has suggested that CCA1 and LHY act as negative regulators through the EE (Alabadi et al., 2001). To test their role in EE:luciferase gene expression, we performed crosses between our EE reporter lines and plants overexpressing either *CCA1* or *LHY*. Levels of luciferase activity were greatly decreased, and rhythms were compromised in *generic\_EE*, *CCR2\_EE*, and *PRR9\_EE* plants that overexpress *CCA1* (Figures 4A to 4C). A similar reduction in luciferase activity and rhythmicity is seen in *PRR9\_EE* plants that overexpress *CCA1* (Figure 4D), despite the differences in phases of bioluminescence conferred by the EE and ME reporters. We found that overexpression of *LHY* also caused a pronounced decrease in luciferase activity in

*generic\_EE* and *CCR2\_EE* plants (data not shown). These data are consistent with the model that CCA1 and LHY inhibit transcription through the EE.

Surprisingly, many EE:luc<sup>+</sup> plants overexpressing *CCA1* showed rhythmic luciferase activity with approximately wild-type periods, albeit with greatly reduced amplitude (Figures 4A to 4C). This raises the exciting possibility that overexpression of *CCA1* masks a functional circadian oscillator rather than causing the central oscillator itself to become arrhythmic. We are currently investigating whether other circadian outputs show low-amplitude rhythms in *CCA1*- and *LHY*-overexpressing plants.

To further examine the role of CCA1 and LHY in EE-mediated gene expression, we twice introgressed two different *CCR2\_EE:luc<sup>+</sup>* reporter lines (Columbia [Col]) into plants null for both *CCA1* and *LHY* (*Landsberg erecta* [Ler]). *CCA1/LHY* knockout plants quickly become arrhythmic upon transfer to constant environmental conditions (Alabadi et al., 2002; Mizoguchi et al., 2002). To compensate for potential ecotype-specific differences, we examined luciferase activity in segregating F2 populations, comparing plants with wild-type rhythms to arrhythmic plants. Robustly rhythmic plants with wild-type periods had more luciferase activity than their arrhythmic siblings, indicating that decreased dosage of *CCA1* and *LHY* caused a decrease in EE:luc<sup>+</sup> activity instead of the expected increase (Figures 4E and 4F). Surprisingly, overexpression and loss-of-function of *CCA1* and *LHY* have similar effects on EE-mediated gene regulation.

To investigate whether one or both of these effects is likely due to direct action of CCA1 and LHY on the EE, we performed a series of electrophoretic mobility shift assays (EMSA) investigating the ability of CCA1 and LHY to bind to wild-type and mutant EE sequences. Extracts from bacteria expressing

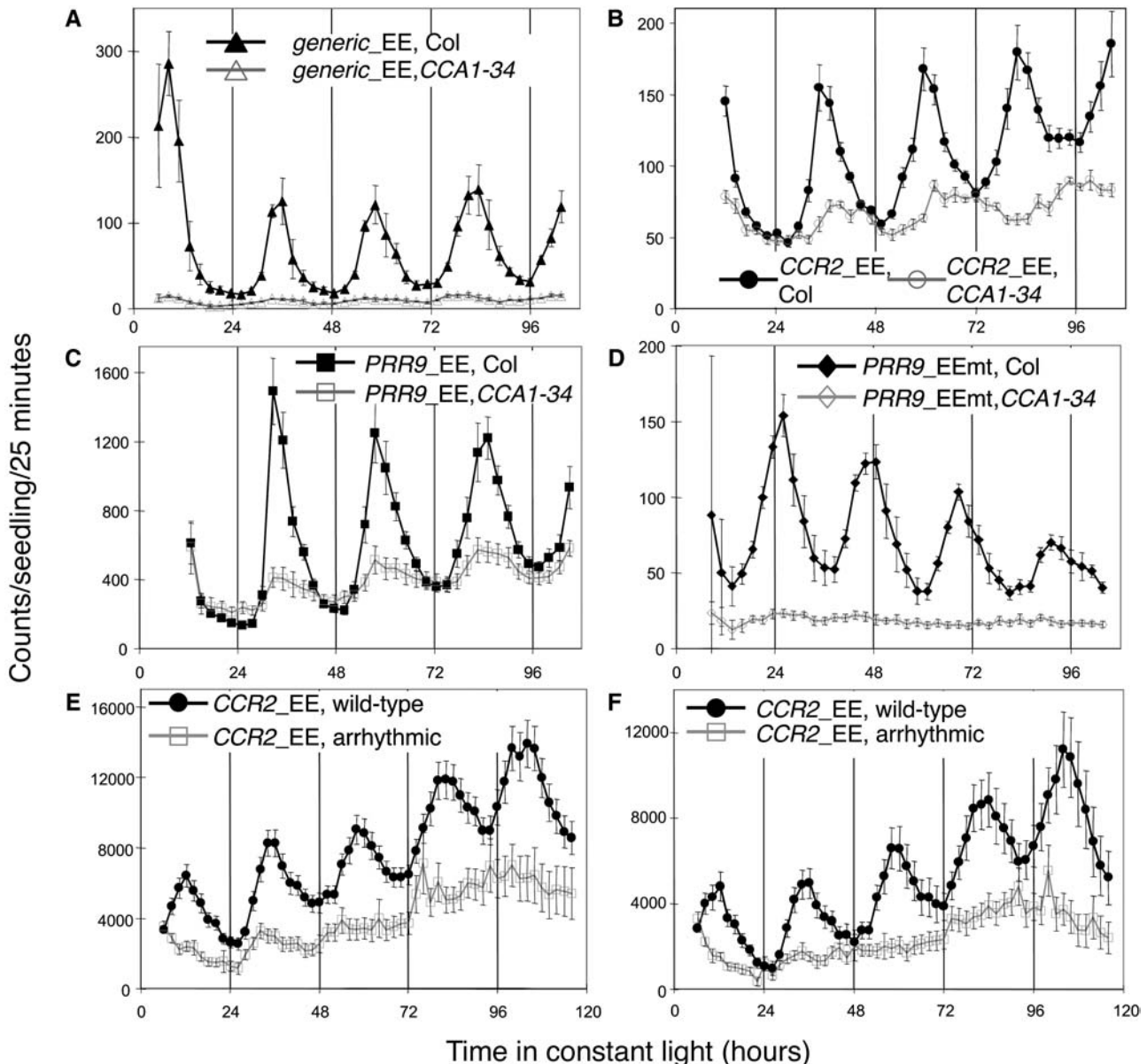


**Figure 3.** Multimerized CBS Confer Evening-Phased Rhythms.

**(A)** One nucleotide of each EE in the *CCR2*- and *PRR9*-derived multimers was mutated from T to A, creating *CCR2\_CBS* and *PRR9\_CBS* multimers driving luciferase expression.

**(B)** T1 plants were assayed as described in Figure 1, except that plants were transferred to constant red light for luciferase assays. Data from each plant were normalized to its median expression level. Averages ( $\pm$  SE) of all plants that returned an RAE (i.e., for which a rhythm was detected) are depicted. The number of plants averaged for each trace is indicated in **(A)**. Data for additional T1 plants are summarized in Table 1.

**(C)** Phase and RAE are plotted for all *CCR2\_CBS* and *PRR9\_CBS* plants for which a rhythmic component of luciferase activity was determined.



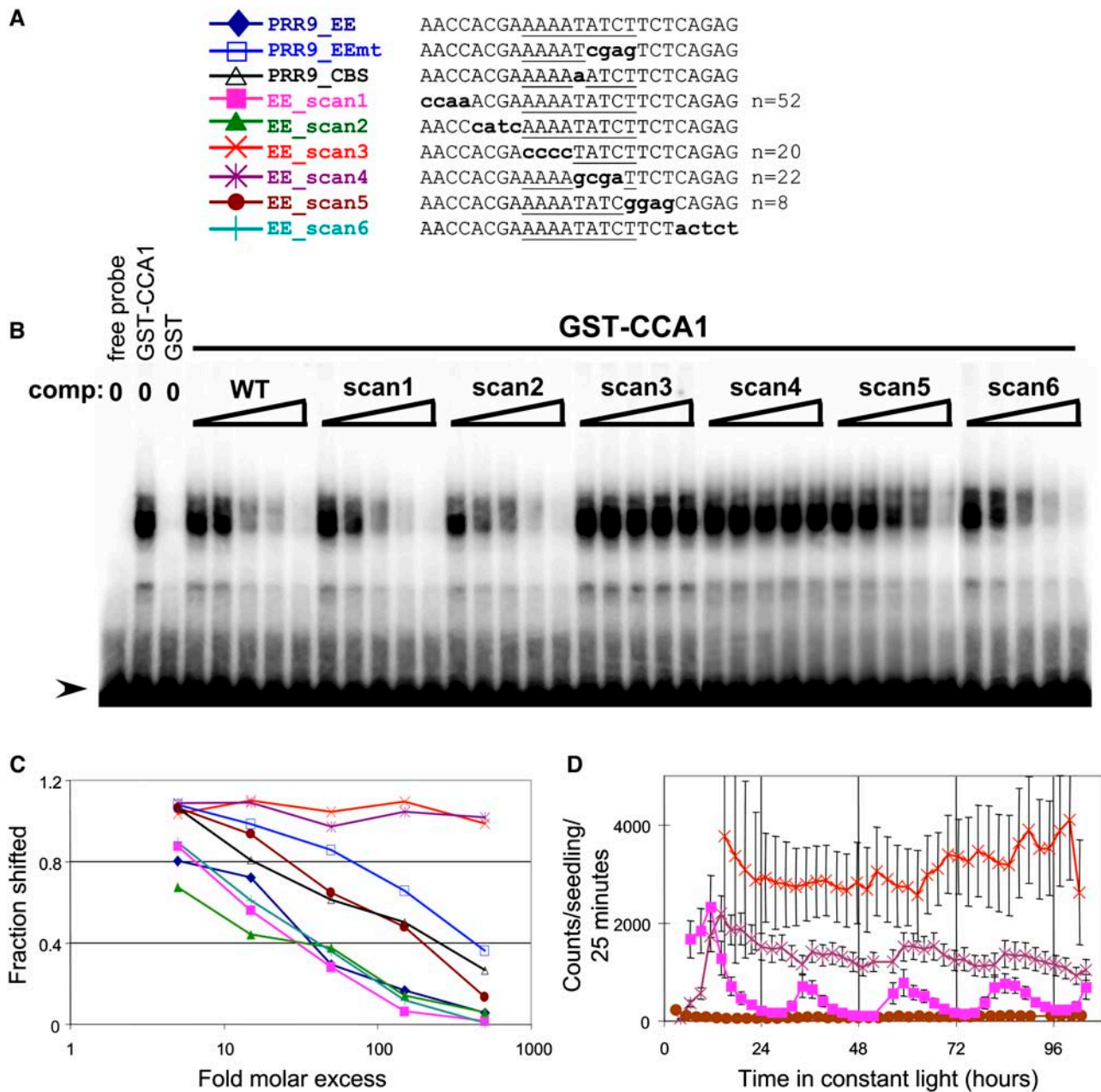
**Figure 4.** CCA1 Acts Both Positively and Negatively via the EE.

(A) to (D) Plants that constitutively overexpress *CCA1* (Wang and Tobin, 1998) were crossed to plants that express luciferase under the control of the *generic\_EE* (A), *CCR2\_EE* (B), *PRR9\_EE* (C), or *PRR9\_EEemt* (D) multimers. F1 progeny and the parental *EE:luc+* lines were assayed as described in Figure 1. The average of between 9 and 18 plants,  $\pm$  SE, is depicted.

(E) and (F) Plants from two different *CCR2\_EE:luc+* lines (Col) were introgressed twice into the *cca1-1 lhy-12* mutant background (*Ler*). F2 populations segregating for the transgene and both mutations were assayed; all plants with wild-type rhythms were compared with all plants with no significant rhythms detected. Twenty-six plants with wild-type rhythms and 24 plants with no detectable rhythms (total population = 130) were compared for (E); for (F), 23 plants with wild-type rhythms and 37 plants with no detectable rhythms (total population = 155) were compared. Averages  $\pm$  SE are shown.

glutathione S-transferase (GST)-*CCA1* or GST-LHY were added to radiolabeled double-stranded oligonucleotides containing one copy of the *CCR2\_EE* sequence. Addition of these extracts, but not extracts from bacteria expressing GST alone, caused the appearance of a DNA species with retarded mobility (Figure 5B; see Supplemental Figures 1 and 2 online). This demonstrates that *CCA1* and LHY bind directly to the EE in vitro, consistent with

previous reports (Alabadi et al., 2001; Farre et al., 2005). Specificity of binding was investigated by competitions with unlabeled DNA fragments. Oligonucleotides containing either the *generic\_EE*, *CCR2\_EE*, or *PRR9\_EE* competed similarly for binding to recombinant *CCA1* or LHY (data not shown). Both *CCA1* and LHY showed greatly reduced affinity for the *generic\_EEemt*, *CCR2\_Eemt*, and *PRR9\_Eemt* sequences (Figure 5C; see



**Figure 5.** Both Positive and Negative Factors Mediate Cycling through the EE.

**(A)** Sequence of EE mutants used in DNA binding experiments or to create luciferase reporter vectors.

**(B)** Extracts from bacteria expressing GST-CCA1 or GST were incubated with radiolabeled double-stranded DNA containing the *CCR2\_EE* sequence. A 5-, 15-, 50-, 150-, or 500-fold molar excess of unlabeled competitor DNA was added to each reaction as indicated. DNA and protein/DNA complexes were separated by nondenaturing gel electrophoresis and visualized using a phosphor imager. The arrowhead indicates unbound probe.

**(C)** Binding curves for each competitor is indicated, based on data from **(B)** and from Supplemental Figure 1 online.

**(D)** Average luciferase expression ( $\pm$  SE) of visible plants is shown for T1 plants transformed with constructs with luciferase expression regulated by the mutant EE sequences indicated in **(A)**. Experiments were performed as described in Figure 1. Plants transformed with the EE\_scan2 and EE\_scan6 constructs have similar expression patterns to the EE\_scan1 plants and have been omitted for clarity. The number of plants averaged is indicated in **(A)**. Data on additional T1 plants are in Table 1.



Supplemental Figures 1 and 2 online; data not shown). To investigate the relative importance of different regions of the EE, we performed scanning mutagenesis across the *PRR9*\_EE sequence and used these mutated double-stranded oligonucleotides as competitors. Sequences with mutations outside the EE (EE\_scan1, EE\_scan2, and EE\_scan6) showed similar affinity as wild-type *PRR9*\_EE sequences (Figures 5B and 5C; see Supplemental Figure 2 online). The EE\_scan3 and EE\_scan4 competitors, with four of the nine EE residues altered, showed essentially no binding to CCA1 or LHY at even 500-fold molar excess over the probe. The EE\_scan5 mutant, with only the last residue of the EE altered, showed an intermediate level of competition, achieving half-maximal competition at ~150-fold molar excess, compared with the half-maximal competition seen at ~25-fold molar excess for wild-type sequences. This affinity is very similar to that of the *PRR9*\_CBS sequence and higher than the affinity of the *PRR9*\_EEmt sequence (Figures 5B and 5C; see Supplemental Figures 1 and 2 online) for CCA1 and LHY. Thus, mutation of sequences computationally defined as the EE disrupts binding of both CCA1 and LHY.

### Positive Regulators of Transcription Must Also Act through the EE

We next wished to investigate the correlation between CCA1 and LHY binding and rhythmicity. Therefore, we made a series of multimerized luciferase reporter constructs corresponding to the *PRR9* scanning mutants used for the competition assays described above. As expected, mutations outside of the EE itself (scan1, scan2, and scan6) had little effect on either luciferase rhythmicity or phase (Figures 5A and 5D, Table 1; data not shown). By contrast, mutations that altered the computationally defined EE (EE\_scan3, EE\_scan4, and EE\_scan5) had a strong effect on rhythms, reducing the percentage of rhythmic seedlings from the 95% seen with wild-type constructs to between 15 and 48% (Figure 5D, Table 1).

Interestingly, these mutations had disparate effects on the levels of luciferase activity. EE\_scan1 plants did not show a significant difference in expression when compared with *PRR9*\_EE plants. Mutations immediately adjacent to the EE (EE\_scan2 and EE\_scan6) reduced expression level by approximately twofold in transgenic plants, a small but significant effect. Mutations within the 5' and middle portions of the EE (EE\_scan3 and EE\_scan4) resulted in plants with significantly increased average luciferase activity: 11- and 6-fold higher than *PRR9*\_EE, respectively (Figure 5D, Table 1). On the other hand, plants transformed with EE\_scan5, in which the last nucleotide of the EE and three flanking nucleotides were mutated, had sixfold lower levels of bioluminescence than *PRR9*\_EE plants, also a highly significant result (Figure 5D, Table 1). Although 95% of visible *PRR9*\_EE plants were rhythmic, less than half of the visible EE\_scan5 plants ( $n = 27$ ) returned a detectable rhythm. This suggests that mutation of the 3' end of the EE altered the binding site of a positive factor that also plays a role in circadian regulation. Therefore, both negatively and positively acting transcription factors bind to the EE and both likely contribute to rhythm generation.

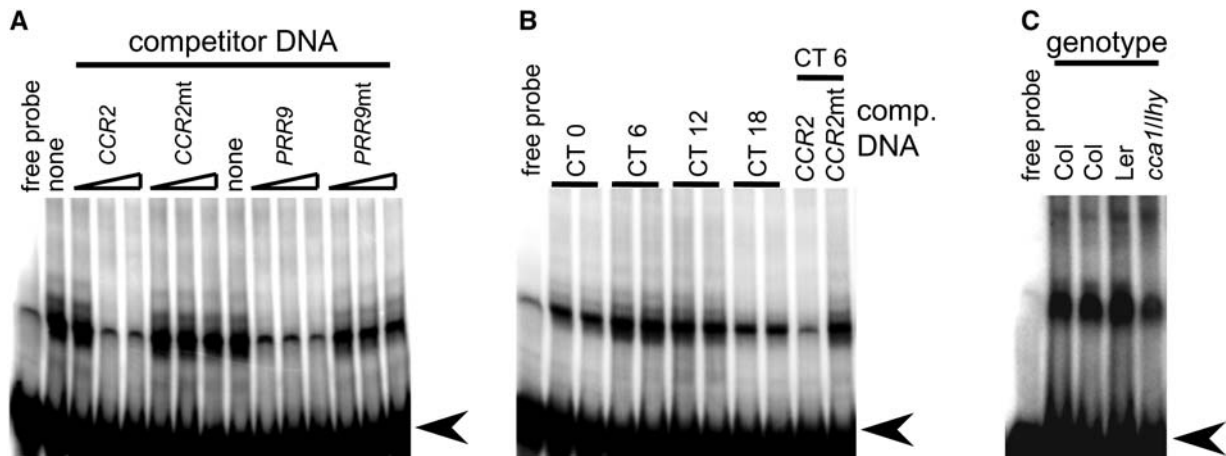
To investigate the possibility that a transcriptional regulator other than CCA1 and LHY might bind the EE, we performed EMSAs using crude Arabidopsis extracts as the source of protein. We found an activity in extracts made from plants harvested in the afternoon (zeitgeber time [ZT] 6, or 6 h after dawn) that showed specific binding to wild-type but not mutant EE sequences (Figure 6A). We investigated whether the abundance of this activity was clock regulated by entraining plants in cycles of 12 h light/12 h dark, then transferring them to constant light and harvesting samples at different times of the subjective day and night. We found that extracts made from samples harvested at CT 6 and CT 12 had much more binding activity than samples harvested at CT 18 and CT 0 (Figure 6B). The peak abundance of this binding activity in the subjective afternoon and evening correlates with the time of peak EE:luc+ expression, suggesting that it might correspond to an activator of transcription. This phase also suggests that the activity is not caused by CCA1 and LHY because their protein levels peak near subjective dawn (Wang and Tobin, 1998; Kim et al., 2003). To test this possibility, we made extracts from plants null for both *CCA1* and *LHY* (Mizoguchi et al., 2002). These extracts had ~50% of the binding activity found in wild-type controls (Figure 6C). This reduction in binding activity was seen in plants harvested in the afternoon (ZT 8) and in plants harvested before dawn (ZT 20) (Figure 6C; data not shown), suggesting that this difference is not simply caused by an alteration in the phase of peak binding activity in the double knockout. The persistence of specific binding activity in the double knockout demonstrates that plant factors other than CCA1 and LHY can bind to the EE and may play a role in its regulation.

## DISCUSSION

### EE and CBS Are Necessary and Sufficient to Confer Evening-Phased Rhythms

The EE was initially defined as a motif overrepresented in the promoters of clock-regulated genes, most frequently those with evening-phased peak expression (Harmer et al., 2000). The EE has been implicated in the circadian regulation of genes important for clock function, such as *TOC1*, *ELF4*, and *GI*, as well as clock output genes (Harmer et al., 2000; Alabadi et al., 2001; Mizoguchi et al., 2002). Thus, the EE plays a role in plants analogous to that of the E-box in animals, which controls the expression of central clock genes, such as *PERIOD* (*PER*) and *TIMELESS* genes in flies and *PER* and *CRYPTOCHROME* genes in mammals, and additionally regulates clock output genes (Ueda et al., 2005). Here, we have shown that four tandem repeats of the EE are sufficient to confer evening-phased gene expression on a reporter gene and that mutation within the computationally defined EE disrupts this cycling. Similarly, four multimerized repeats of the *PER* E-box are sufficient to confer *PER*-like expression on a reporter gene in *Drosophila* (Darlington et al., 2000).

Specific sequences flanking the EE are not required for its function, as shown by the ability of the *generic*\_EE construct to confer evening-phased rhythms on luciferase. However, the



**Figure 6.** A Clock-Regulated Activity in Plant Extracts Binds the EE.

**(A)** Extracts made from Arabidopsis seedlings (Col) harvested at ZT 6 were incubated with radiolabeled double-stranded DNA containing the *CCR2*\_EE sequence. A 5-, 15-, or 50-fold molar excess of unlabeled competitor DNA was added to each reaction as indicated. Protein/DNA complexes were separated by nondenaturing gel electrophoresis and visualized using a phosphor imager. Specificity of binding is shown by the ability of fragments with wild-type EE sequences, but not fragments in which these sequences are altered, to compete for binding to proteins in the extracts.

**(B)** Arabidopsis seedlings (Col) were harvested at the indicated times of the subjective day and night, and extracts were made. Binding assays were performed in duplicate. A 50-fold molar excess of the indicated competitor DNAs was used.

**(C)** Extracts were made from Col, *Ler*, and *cca1-1 lhy-12* (*Ler*) plants (all harvested at ZT 8) and binding assays performed as described in **(A)**. The arrowheads indicate unbound probe.

spacing between the EE is important: a construct with only three nucleotides between each of four EE resulted in transgenic plants with no visible luciferase activity ( $n = 26$ ; data not shown). The three rhythmic EE constructs described here each have 16 nucleotides between the EEs, close to the distance between two of the EEs in the native *CCR2* promoter. However, we found that two multimerized copies of the EE separated by 16 bp are not sufficient to confer rhythms on a luciferase reporter gene (data not shown).

Recent improvements in genome annotation have allowed the unequivocal identification of the promoter regions of 399 genes whose expression was judged to be clock regulated in a microarray study (Harmer et al., 2000). Examination of the promoter regions of these genes (defined as the 1 kb upstream of the transcriptional start site) revealed 84 occurrences of the EE upstream of 70 different genes, and 61% of the EE were in the forward orientation and 39% were in the reverse orientation. No correlation was observed between expression pattern and orientation of the EE. By contrast, 47 (67%) of the genes with EE in their promoters had evening-phased (CT 8 or CT 12) expression, compared with 171 (43%) total cycling genes with this phase of expression. This statistically significant ( $P = 0.0009$ ,  $\chi^2$  test) overrepresentation of EE in the promoters of evening-phased genes is consonant with the evening phase conferred by multimerized EE. However, 60 of the 70 EE-containing promoters have only a single EE, despite our finding that two multimerized EEs are not sufficient to confer rhythmic gene expression. This suggests that in native promoters other elements act with the EE to achieve clock-regulated gene expression. In addition, more than half of the evening-phased genes do not have an EE in their promoter regions, indicating that other enhancer elements can also confer evening-phased gene expression.

A motif very similar to the EE, the CBS, has been proposed to impart dawn-phased rhythms (Michael and McClung, 2002). The CBS is found in the promoters of many clock-regulated genes with peak expression around mid-day (Wang et al., 1997; Piechulla et al., 1998; Michael and McClung, 2003). We therefore examined the distribution of CBS sequences in the promoters of the 399 clock-regulated genes described above. There was no significant skewing of the phases of peak expression for either the 24 genes with a long CBS (AAAAAATCT, or the reverse complement) or the 57 genes with a short CBS (AAAAATCT, or the reverse complement) relative to the other clock-regulated genes, suggesting that in native promoters the CBS does not act alone to specify circadian phase.

Michael and McClung (2002) showed that a 200-bp fragment of the *CAT3* promoter, containing a single EE, conferred evening-phased rhythms in luciferase activity (in both constant darkness [DD] and constant light [LL]). However, mutation of the EE to a CBS resulted in transgenic plants that were arrhythmic in LL but had dawn-phased luciferase rhythms in DD. By contrast, we found that constructs containing multimerized CBS confer the same evening-phased rhythms in LL (Figure 4, Table 1) and DD (data not shown) as do multimerized EE. Fewer CBS:luc+ than EE:luc+ plants were rhythmic, as also reported for CBS *CAT3*:luc plants relative to the EE *CAT3*:luc plants. This reduced frequency of rhythmic plants may be due to the reduced affinity of CCA1 and LHY for the CBS as compared with the EE (Figure 5C; see Supplemental Figures 1 and 2 online). Why do we find that the CBS confers evening-phased rhythms while Michael and McClung (2002) found it to confer dawn-phased rhythms? The discrepancy may be either because multimerized CBS motifs produce a different phase than a single CBS or that other motifs

present in the *CAT3* promoter modify CBS function to change the phase from dusk to dawn or perhaps produce morning-phased rhythms on their own. Similar discrepancies have been reported in studies using either multimerized E-boxes or a single larger E-box-containing promoter fragment (Darlington et al., 2000; Lyons et al., 2000).

### A Distinct Promoter Motif Confers Morning-Phased Rhythms

Serendipitously, we found that small changes in the sequences of our enhancer constructs resulted in an almost 180° change in the phase of luciferase activity in transformed plants: *PRR9\_EE* plants have an evening phase, whereas *PRR9\_EE*mt plants have a morning phase. Notably, the mutation that produces dawn-phased rhythms in *PRR9\_EE*mt plants abrogates the ability of the *generic\_EE* and *CCR2\_EE* constructs to confer rhythmicity, indicating that *PRR9*-specific flanking sequences are responsible for this morning-phased rhythmicity. Because two constructs in which the last four nucleotides of the EE in the *PRR9\_EE* sequence were altered to different sequences (*PRR9\_EE*mt and *ME\_scan4*) both confer morning-phased rhythms, we conclude that a functional ME exists in the wild-type *PRR9\_EE* sequence. ME activity is somehow masked or modified by the EE in our multimerized constructs, but mutation of the EE allows ME function to be observed.

Scanning mutagenesis of the *PRR9\_EE*mt sequence revealed that 12 nucleotides of this fragment (AACCACGAAAAT) are essential for rhythm generation, whereas downstream sequences are dispensable for rhythmicity but important for phase consolidation (Figure 2, Table 1). A region we find to be essential for ME function (AACCAC) is also present within a motif that is overrepresented in the promoters of clock-regulated genes (CACTAACCAC) (Hudson and Quail, 2003). Thus, the experimentally defined ME may play a more general role in circadian regulation of transcription. However, inspection of the promoters of 399 clock-regulated genes did not reveal preferential location of any of the three above sequences or their reverse complements upstream of genes with any particular phase. This suggests that either we have not yet identified the true ME consensus sequence or that other motifs are also required to confer morning-specific regulation in native promoters.

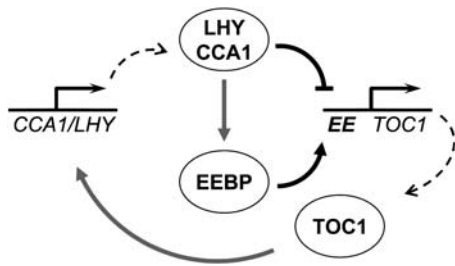
Studies of the native *PRR9* promoter have shown that one copy of the region containing the EE and ME does not confer rhythmic gene expression (Ito et al., 2005). However, a small region of the *PRR9* promoter, upstream of both the ME and the EE, confers evening-phased expression on a reporter gene in light/dark cycles, but this cycling quickly damps in LL (Ito et al., 2005). It is striking that the ME, EE, and the unknown sequence identified by Ito et al. (2005) generate either dawn- or evening-phased rhythms when isolated. This raises the possibility that in the native promoter these motifs interact to generate the observed morning phase of *PRR9* gene expression, intermediate between dawn and dusk. Bioinformatic analysis suggests that other, as yet unknown, motifs are involved in this process: EE-containing clock-regulated promoters that also have an ME show no skewing in phase distribution when compared with promoters that have an EE alone.

### Dawn- and Dusk-Phased Rhythms Are the Product of Both Positive and Negative Factors

Our identification of 25-bp fragments that confer dawn- and dusk-specific rhythms when multimerized allowed us to use scanning mutagenesis to study the types of *trans*-acting factors that bind to these motifs. Mutation of the 5' half of the ME greatly reduced luciferase activity and the percentage of rhythmic seedlings. Thus, positive acting transcription factor(s) bind to this region and are required for rhythmicity. These activating factors are unlikely to be CCA1 or LHY because these factors bind poorly to *PRR9\_EE*mt in vitro (Figure 5C; see Supplemental Figures 1 and 2 online) and their overexpression causes a reduction rather than an increase of luciferase activity in *PRR9\_EE*mt:luc+ plants (Figure 6; data not shown). Mutations in the 3' half of the ME affect phase consolidation and average expression levels, with both the *ME\_scan4* and *ME\_scan5* mutations causing more variability in phases of peak expression and the *ME\_scan4* mutation increasing luciferase activity (Table 1, Figure 2E). We therefore suggest that this region is important for accurate phasing of rhythms and that a negative regulator of transcription is involved in this process. The identities of the transcription factors (positive or negative) that confer morning-phased rhythms via the ME are presently unknown.

Scanning mutagenesis of the *PRR9\_EE* promoter fragment suggests that both positive and negative factors regulate the EE as well. Mutations within the 5' portion of the EE increase luciferase activity, and a mutation at the 3' end decreases luciferase activity (Figure 5, Table 1). As previously proposed, the negative factors are most likely to be the Myb factors CCA1 and LHY (Alabadi et al., 2001) because EE-mediated gene expression is antiphasic to that of CCA1 and LHY and is reduced when either gene is overexpressed. Furthermore, the binding affinity of CCA1 and LHY for mutated EE sequences in vitro correlates with the ability of these mutants to confer rhythms on a reporter gene in planta (Figures 4 and 5; see Supplemental Figures 1 and 2 online). The identity of the positive factor(s) is unknown, but we have found an EE binding activity in plant extracts whose abundance is clock regulated (Figure 6B). This activity peaks in the mid to late subjective day, consistent with it representing an activator of transcription through the EE. Notably, its abundance is decreased in plants deficient for both *CCA1* and *LHY* (Figure 6C), suggesting that it forms part of a secondary loop within an oscillator network (Figure 7). This reduction of evening-phased binding activity in plants mutant for both *CCA1* and *LHY* may explain the reduction of *CCR2\_EE*:luc+ activity seen in plants with reduced dosage of *CCA1* and *LHY* (Figures 4E and 4F). We are currently using biochemical and genetics approaches to identify this factor(s). Our data suggests that positive- and negative-acting factors work together through the EE to enhance the amplitude of cycling and produce robust well-consolidated rhythms.

Our in vitro binding studies with CCA1 and LHY also support the idea that an activator of transcription binds *PRR9\_EE*. The *PRR9\_CBS* and the *EE\_scan5* mutant sequences have very similar affinities for CCA1 and LHY, at least in vitro (Figure 5; see Supplemental Figures 1 and 2 online). However, the *EE\_scan5* mutation has a more severe effect on the ability of the EE to



**Figure 7.** A Secondary Loop Acts within the Plant Central Clock.

CCA1 and LHY bind to the EE in many clock-regulated promoters, acting to repress transcription. They also, directly or indirectly, increase expression and/or activity of an EE binding protein (EEBP) that binds to the EE and acts as a transcriptional activator. TOC1 protein, directly or indirectly, promotes expression of *CCA1* and *LHY*. Steps thought to occur directly are indicated by black arrows; steps that may be direct or indirect are indicated by gray arrows.

confer rhythmic gene expression than does the *PRR9\_CBS* mutation (Table 1; data not shown). In addition, the average luciferase expression level of *EE\_scan5:luc+* plants is significantly lower than those of *PRR9\_CBS:luc+* plants (data not shown). These data suggest that the more severe phenotype seen in the *EE\_scan5:luc+* plants is due to the disruption of a binding site for a transcriptional activator in addition to disruption of *CCA1/LHY* binding.

There are precedents for both positive and negative regulators of transcription binding to the same promoter motif. In *Arabidopsis*, the closely related basic leucine zipper (bZIP) factors TGA4 and TGA5 act to promote and repress, respectively, expression mediated by the *octopine synthase/activation synthase-1* element (Foley and Singh, 2004). This type of mechanism also regulates expression of core clock genes in animals. Circadian regulation of *Drosophila Clock (dClk)*, a basic helix-loop-helix protein, is achieved through the antagonistic actions of two bZIP factors, *Vrille (Vri)* and *Par Domain Protein 1 (Pdp1)*. These factors compete for binding to the same element in the *dClk* promoter, with the repressor (*Vri*) showing antiphasic expression to *dClk* and the activator (*Pdp1*) showing peak expression slightly before *dClk* (Cyran et al., 2003; Glossop et al., 2003). Mammals also use both positive and negative factors to regulate a central clock gene in the basic helix-loop-helix family. But surprisingly, these factors belong to the orphan nuclear receptor family, and the gene under regulation is the *dCLK* paralog *Bmal1* rather than the mammalian *Clk* ortholog. The retinoic acid-related orphan receptor Rev-erb  $\alpha$  represses while its paralog Rora activates expression of *Bmal1*, both acting competitively through the same site in the *Bmal1* promoter (Preitner et al., 2002; Sato et al., 2004). Similarly, two clock-regulated and antiphasic bZIP family proteins (Dbp and E4bp4), one an activator and one a repressor of transcription, bind competitively to D-box sites in the promoters of mammalian clock genes to help drive high-amplitude rhythmic expression (Mitsui et al., 2001; Ueda et al., 2005). Thus, flies and mammals use different types of transcription factors with both positive and negative functions to form secondary feedback loops that

enhance the precision and stability of the primary feedback loops driving clock function. Our data suggest that plants use a similar mechanism to regulate gene expression through the EE.

### Flanking Regions Modify the Function of Clock Motifs

Although we have shown that the EE is necessary and sufficient to confer evening-phased rhythms, our data also support the idea that nearby sequences can modulate its function, affecting rhythmicity, phase, amplitude, and expression level.

Artificially multimerized EE confer approximately the same evening phase regardless of flanking sequences (Table 1). However, a 1.6-kb region of the *CCR2* promoter (containing three EE motifs) fused to luciferase produces bioluminescence rhythms with peaks at CT 12 (Strayer et al., 2000; data not shown), earlier than the CT 13.4-phased rhythms produced by the *CCR2\_EE* construct. An even more striking phase difference is seen when comparing the phase of the endogenous *PRR9* transcript, which peaks around CT 4 (Matsushika et al., 2000), and *PRR9\_EE:luc+* plants, which peak at CT 12.8. These data suggest that other motifs within a native promoter can modify the activity of the EE to produce different phases.

Additional evidence that flanking regions affect rhythmicity comes from a comparison of plants transformed with the *generic\_EE:luc+* and *CCR2\_EE:luc+* constructs. *CCR2\_EE:luc+* plants display stronger rhythms in luciferase activity, with both higher amplitude and more robust cycling (Figure 1; data not shown) than *generic\_EE:luc+* plants. One possible explanation for this finding comes from the observation that mutation of the last four bases of the EE causes a 10-fold increase in average levels of luciferase activity in *generic\_EE* plants but a 10-fold decrease in luciferase activity in *CCR2\_EE* plants (Table 1). In combination with our other results, this suggests that the *generic\_EE* sequence is bound solely by repressors of transcription (*CCA1* and *LHY*) but that the *CCR2\_EE* sequence is bound by both repressors and activators, resulting in more robust rhythms. Our analysis of EE-containing promoters hasn't revealed any consensus sequences outside the EE itself, so both the identity of the putative activator and non-EE sequences important for its binding remain to be determined.

The previously proposed model of the central oscillator in plants suggests that *CCA1* and *LHY* negatively regulate *TOC1* expression via the EE, and *TOC1* protein in turn contributes to positive regulation of *CCA1* and *LHY* expression (the outer loop in Figure 7). According to this model, genes such as *TOC1* that are regulated via an EE should show a decrease in expression in plants overexpressing *CCA1* or *LHY* and an increase in expression in plants deficient for both *CCA1* and *LHY*. *TOC1* indeed shows increased expression in plants null for both Myb factors (Mizoguchi et al., 2002), but *TOC1* levels are intermediate in *CCA1*-overexpressing plants (Matsushika et al., 2002). Also inconsistent with the simplest model, *CCR2*, an evening-phased gene whose rhythmic expression depends upon a functional EE (Harmer et al., 2000), has reduced rather than increased expression in *CCA1/LHY* null plants (Mizoguchi et al., 2002; Kim et al., 2003) and relatively unchanged expression levels in plants

overexpressing *LHY* (Suarez-Lopez et al., 2001; Kim et al., 2003). These discrepancies may in part be explained by a transcriptional activator that binds to the EE but whose expression is dependent upon *CCA1* and *LHY*. Knockout of both *CCA1* and *LHY* would lead directly to a loss of these transcriptional inhibitors and indirectly to a decrease in a transcriptional activator that also binds to the EE (Figure 7). Depending upon the relative affinities of the Myb factors and the putative transactivator for a particular EE-containing promoter, this might lead to either an increase or decrease in expression of that gene in *CCA1/LHY* knockout plants. We predict that the putative transcriptional activator identified in this study acts as part of a secondary feedback loop to enhance precision and robustness in the plant circadian clock.

## METHODS

### Plasmid Construction

All luciferase constructs were made by inserting the indicated enhancer elements into the *SacI/XhoI* sites of pATM-Nos, upstream of the -101/+4 fragment of the NOS minimal promoter (Puente et al., 1996) and modified firefly luciferase (*luc+*) (pATM-Nos provided by C. Andersson, unpublished data). Four oligonucleotides, each encompassing two of the four repeated sequences, were annealed to each other to create the 107-bp artificial enhancer sequences. The *generic\_EE* sequence was chosen by aligning all 46 EE identified in the promoters of clock-regulated genes by Harmer et al. (2000) and selecting the nucleotide that was least common at each position flanking the EE. The *CCR2\_EE* sequence is based upon the region -77 to -53 bp upstream of the 5' untranslated region, and the *PRR9\_EE* sequence is based upon the region -136 to -113 bp upstream of the 5' untranslated region. See Methods in the supplemental data online for the complete sequences of the synthetic enhancers.

### Plant Materials and Growth Conditions

Plasmids were transformed into the Col-0 accession using the floral dip method (Clough and Bent, 1998). Except where indicated, seedlings were grown on MS medium (Gibco BRL, Cleveland, OH) with 0.8% agar and 3% sucrose. Selection for transgenic T1 plants was performed using kanamycin (50  $\mu\text{g}/\text{mL}$ ). The *CCA1*-overexpressing line 34 (Col), the *LHY*-overexpressing line *lhy-1* (*Ler*), and the *cca1-1 lhy-12* double mutant (*Ler*) have been previously described (Schaffer et al., 1998; Wang and Tobin, 1998; Mizoguchi et al., 2002). For comparison of EE:*luc+* activity in wild-type plants and plants overexpressing *CCA1*, F1 progeny of the appropriate crosses were examined (all F1 plants had long hypocotyls). To determine the effect of *LHY* overexpression, EE:*luc+* plants were crossed to *lhy-1* and to *Ler* plants and the two types of F1 progeny compared. To determine EE:*luc+* activity when both *CCA1* and *LHY* gene dosage was reduced, two independent *CCR2\_EE:luc+* lines (Col) were introgressed two times into the *cca1-1 lhy-12* (*Ler*) background. F2 families segregating for the transgene and both mutations were monitored for luciferase activity. For luciferase assays, plants were grown under 12-h-light (cool white fluorescents, 50 to 60  $\mu\text{mol m}^{-2} \text{s}^{-1}$ )/12-h-dark photoperiods for 5 to 7 d before being released into constant conditions for rhythm analysis. Plants for EMSA extracts were grown for 11 d in light/dark cycles as described above and either harvested at the indicated ZT or transferred to constant white light and harvested at the indicated CT.

### Imaging Assays and Analysis

Seedlings were sprayed with 2.5 mM luciferin (Biosynth International, Naperville, IL) and then placed in either constant white light (cool white

fluorescents, 50 to 60  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) or constant red light (peak wavelength 670 nm, 15-nm half-peak bandwidth; 60  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ; Quantum Devices, Barneveld, WI). Plants assayed in constant white light were imaged as previously described using a Hamamatsu VIM CCD camera (Millar et al., 1995), whereas plants assayed in constant red light were imaged for 15 min every 2 h using an ORCA II ER CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan). Background subtraction was used for ORCA II ER image analysis. Plants were defined as visible if they returned an average of 12 counts/seedling/25 min or greater using the VIM camera or an average of 825 counts/seedling/15 min or greater (after background subtraction) using the ORCA II ER camera. Estimates of period, phase, amplitude, and RAE were obtained using fast Fourier transform nonlinear least squares analysis (Plautz et al., 1997), with periods between 20 and 28 h considered to be within the circadian range. Plants that returned an RAE of <1.0 were considered to have rhythmic luciferase activity. (The relative abilities of the different constructs to confer rhythmic luciferase activity were similar when we arbitrarily defined rhythmic plants as those with RAE <0.5.) To compare phases of peak expression, we compensated for differences in period length in individual plants by converting the observed time of peak expression to CT (= observed peak  $\times$  24/observed period). All phase plots were generated by graphing period on the circumference of a 24-h clock face versus RAE along the radius, with RAE = 1 at the center and RAE = 0 on the periphery. Phase plots, average phase, and phase standard deviation were generated using the circular package of the computer language R (<http://www.R-project.org>) and custom functions. For determining average luciferase activity conferred by each construct, plants that were drug resistant but not detectable with the CCD camera were assigned an average luciferase activity equivalent to background levels. All drug-resistant plants generated with each construct were then averaged over each entire time course and these averages compared using the Kruskal-Wallis rank sum test, with Bonferroni correction for multiple testing where appropriate. To compare *CCR2\_EE:luc+* levels in plants with functional *CCA1* and/or *LHY* to those deficient for these genes, the F2 populations segregating for *cca1-1* and *lhy-12* were divided into plants with robust rhythms and wild-type period (RAE < 0.5; 22.5 < period < 24.5 h) and plants with no significant rhythm detected (these plants did not return an RAE). All imaging experiments were performed a minimum of three times with similar results.

### EMSA

Gel retardation assays were performed using extracts of *Escherichia coli* induced to express GST, GST-*CCA1*, or GST-*LHY* as the source of recombinant protein. GST-*CCA1* was generated using a full-length *CCA1* cDNA (amplified from a cDNA kindly provided by Carol Andersson using the primers 5'-GTGTAGAGGAGCGAATTCATGGAGA-3' and 5'-GCCGCGCCTAGCTTGAGTTTCCAACCG-3') inserted into the *EcoRI* and *NotI* sites of pGEX-4T1. The pGEX-*LHY* construct, containing the full-length *LHY* cDNA, was the kind gift of Tom Schultz. The empty pGEX-4T1 vector was used to generate GST alone. Saturated cultures of BL21 harboring the pGEX-4T1, pGEX-*CCA1*, or pGEX-*LHY* plasmid were diluted 1:100 in M9 media, grown at 37°C to an OD<sub>600</sub> of 0.1, and isopropyl- $\beta$ -D-thiogalactopyranoside was added to 0.1 mM. After an additional 4 h at 37°C, the cultures were harvested and resuspended (resuspension buffer = 20 mM Hepes, pH 7.2, 75 mM KCl, 10% glycerol, 0.1 mM EDTA, 0.1 mM PMSF, 2.5 mM DTT, and 1 $\times$  Complete protease inhibitor cocktail; Roche, Indianapolis, IN) and the cells disrupted using a probe sonicator. After a high-speed spin, the supernatants were collected, aliquotted, frozen in liquid nitrogen, and stored at -80°C. Protein gel blot analysis was used to estimate the amount of GST fusion protein in each extract. Plant whole-cell extracts were made by first suspending ground plant tissue in homogenization buffer (15 mM Hepes, pH 7.6, 40 mM KCl, 0.1 mM EDTA, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 0.1 mM

PMSF, and 1× Complete protease inhibitor cocktail) and then adding  $\text{NH}_4\text{SO}_4$  to 0.4 M. Insoluble components were pelleted by ultracentrifugation, and solid  $\text{NH}_4\text{SO}_4$  was then added to the supernatant to 90% saturation. Proteins were pelleted by centrifugation and then resuspended in resuspension buffer (20 mM Hepes, pH 7.6, 40 mM KCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.1 mM PMSF, and 1× Complete protease inhibitor cocktail). Total protein concentrations were determined after dialysis with resuspension buffer. The probes and competitor DNA fragments used in these assays were generated by annealing two oligonucleotides together (see Supplemental Methods online for these sequences). The double-stranded oligonucleotides were radiolabeled using Klenow fragment (New England Biolabs, Beverly, MA). Either bacterial cell extracts containing ~2 fmoles of the indicated GST fusion protein or 15  $\mu\text{g}$  of total plant cell protein were incubated with 8 fmoles of the appropriate radiolabeled probe in reaction buffer [20 mM Hepes, pH 7.2, 80 mM KCl, 0.1 mM EDTA, 10% glycerol, 2.5 mM DTT, 0.07  $\mu\text{g}$   $\mu\text{L}^{-1}$  BSA, 8 ng  $\mu\text{L}^{-1}$  poly (dl-dC)] and the appropriate unlabeled competitor DNA (competitor DNA was added at the indicated amounts). Reactions were incubated for 15 min at room temperature and then resolved by electrophoresis on 5% nondenaturing polyacrylamide gels. After drying, gels were imaged using a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA). All EMSA experiments were performed a minimum of three times with similar results.

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